Relevância fenotípica dos polimorfismos do gene MDR nas doenças hematológicas

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Ao Paulo: meu companheiro de jornada.

À Beatriz e Carolina: a minha vida.

INDÍCE

LISTA DE ABREVIATURAS

ADN – ácido desoxirribonucleico
AloTMO – alotransplante de medula óssea
ASR – taxa ajustada à idade (age specific rates)
ATMO – autotransplante de medula óssea
ATP – adenosina trifosfato
BF 12 – idarrubicina, altas doses citarabina e etoposideo
BFM 95 – ciclofosfamida, citarabina (endovenosa e intratecal), metotrexato (endovenosa e intratecal), vincristina, ifosfamida, etoposideo, doxorrubicina, vindesina e dexametasona
CALGB – grupo B de cancro e leucemia [1]
CBF – fator de ligação ao núcleo (core binding factor)
CEBPA – CCAAT / potenciador proteína de ligação alfa (CCAAT/enhancer-binding protein alpha)
CD 20 – antigénio linfócito B
CHOP – ciclofosfamida, vincristina, doxorrubicina e prednisolona
EBV – virús Epstein-Barr
EDTA – ácido etilenodiamino tetra-acético
EHW – equilibrio de Hardy Weinberg
EUA – Estados Unidos da América
FLIPI – Índice de prognóstico internacional para Linfoma de centro folicular
FLT3-ITD – duplicação interna tandem do gene FLT3
HAART – terapêutica antirretrovirica altamente ativa
HHV8 – virús herpes humano 8
HP – *Helicobacter pylori*
HTLV1 – Vírus linfotrópico da célula humana do tipo 1
HyperCVAD – ciclofosfamida, vincristina, doxorrubicina, dexametasona, metotrexato (endovenoso e intratecal), citarabina (endovenoso e intratecal)
IPI – Índice de prognóstico internacional
IPO-Porto – Instituto Português de Oncologia do Porto
iTC – inibidor tirosina-cinase
kDa – kilo Dalton
LA – leucemia aguda
Linker – daunorrubicina, vincristina, prednisolona, L-asparaginase, teniposideo, citarabina, metotrexato (endovenoso e intratecal), 6-mercaptopurina, irradiação craniana
LLA – leucemia linfóide aguda
LMA – leucemia mielóide aguda
LNH – Linfoma não-Hodgkin
LNH DGCB – Linfoma não-Hodgkin difuso de grandes células B
LNH NK/T nasal – Linfoma não-Hodgkin NK/T nasal
LPA – Leucemia aguda promielocítica
MDR1 – gene da resistência múltipla a drogas
MIPI – Índice de prognóstico internacional para Linfoma de células do manto
MLL – gene leucemia linhagem mista (mixed-lineage leukemia gene)

mARN – ácido ribonucleico mensageiro
NA – não alcançado
NMP1 – nucleofosmina
OMS – organização mundial de saúde (WHO)
OR – razão de proporções de risco (odds ratio)
PCR – reação em cadeia de polimerase (polimerase chain reaction)
P-gp – P-glicoproteína
Protocolo "7+3" – citarabina e idarrubicina
RT-PCR – reação em cadeia de polimerase em tempo real (real time polimerase chain reaction)
TTP – tempo até progressão (time to progression)
SG – sobrevida global
SNC – sistema nervoso central
SNP – polimorfismo (single nucleotide polymorphism)
SWOG – grupo oncológico sudoeste (southwestern oncology group)

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RESUMO

Introdução e objetivos: a influência dos polimorfismos do gene MDR1 em risco e sobrevida nas doenças hematológicas malignas é incerto. Os objetivos principais deste estudo são avaliar a prevalência dos polimorfismos do gene MDR1 na população portuguesa, correlacioná-los com a sobrevida e definir o perfil farmacogenómico adequado.

Materiais e métodos: foi efectuado um estudo que analisa 175 doentes com leucemia aguda e linfoma não-Hodgkin. Para validar os nossos resultados estudamos a relação entre o genótipo e a incidência e prevalência destas patologias em diferentes populações.

Resultados: observamos que o polimorfismo C1236T influencia o risco para o aparecimento de Leucemia aguda na população Portuguesa (T vs C: *p*= 0,026 OR 0,49 95% CI 0,24-0,97; CC vs TT: *p*= 0,030 OR 7,37 95% CI 0,94-156,8). Quando analisamos um grupo de populações Europeias e asiáticas constatamos a existência de correlação significativa entre a incidência e prevalência de Leucemia Aguda e a expressão do genótipo 1236CC. Foi também observada a correlação entre o genótipo 3435CC e risco de morte em casos de Linfoma não-Hodgkin. Definiu-se um novo modelo preditivo de sobrevida para ambas as patologias, ao adicionar aos factores de prognóstico conhecidos os genótipo dos polimorfismos do gene MDR1. Nos casos de Leucemia aguda o índice C (definido por Harrel et col) era de 0,791 com a utilização apenas de características clinicas. Com a junção das características genotípicas passou para 0,797.

No caso dos Linfomas não-Hodgkin o índice C subiu de 0,835 para 0,911 ao acrescentar as variáveis genéticas às clinico-patológicas.

Conclusões e discussão: Os polimorfismos do gene MDR1 influenciam quer o risco quer a sobrevida nas doenças hematológicas malignas. Ambos os polimorfismos estudados influenciam a função da p-glicoproteína: o genótipo 1236CC altera o enrolamento da proteína e a sua capacidade de se ligar ao substrato, o genótipo 3435CC aumenta a expressão da p-glicoproteína. Quando a p-glicoproteína está disfuncionante pelo efeito do genótipo 1236CC é incapaz de expulsar algumas substâncias do interior da célula o que pode originar o efeito tóxico de alguns compostos, contribuindo para o seu papel na leucemogénese. Quando a P-glicoproteína está sobrexpressa (resultado do genótipo 3435CC) tanto os fármacos como alguns tóxicos são mais rapidamente eliminadas do espaço intracelular diminuindo o seu efeito e potencial ação terapêutica. Neste estudo definimos também novos modelos preditivos baseados em variáveis clinicas associadas a variáveis genéticas.

ABSTRACT

Background and aims: The influence of MDR1 gene polymorphisms in risk and outcome for haematological malignancies remains unclear. The main purposes of this study are to determine the prevalence of MDR1 gene polymorphisms in this particular population, correlate these polymorphisms with disease outcome and to define a pharmagenomic profile.

Materials and methods: We conducted a study analyzing 175 Portuguese acute Leukemia and non-Hodgkin Lymphoma patients. To validate our results we analyzed the relation between genotype expression and disease (AL an NHL) incidence and prevalence rates in several populations.

Results: We observed that C1236T polymorphism influences risk for Acute Leukemia development in the Portuguese population (T vs C: *p*= 0,026 OR 0,49 95% CI 0,24-0,97; CC vs TT: *p*= 0,030 OR 7,37 95% CI 0,94-156,8). When we analyzed a group of European and Asian populations, we found a significant correlation between the incidence and prevalence rates of acute leukemia and 12136CC genotype expression. The genotype 1236CC also influences death risk in both acute leukemia and non-Hodgkin Lymphoma. We also observed that in this population genotype 3435CC influences death in non-Hodgkin lymphoma. We were also able to define new predictive models in both diseases, adding MDR1 genotype to previously defined prognostic variables. In acute leukemia the C index (defined by Harrel et col.) was 0,791 with only clinical features and reached 0,797 when genetic features were added. In non-Hodgkin lymphoma cases the C index switched from 0,835 to 0,911 whith the association of genetic variables.

Conclusions: We conclude that MDR1 gene polymorphisms influence both risk and outcome for hematological malignancies. The studied polymorphisms influence in p-glycoprotein function: 1236CC genotype changes protein folding and substrate binding capacity and 3435CC genotype increases protein expression. When p-glycoprotein is dysfunctional because of 1236CC genotype, the disability of extrude compounds from the inside of the cell, what may lead to the toxic influence of some substances, contributing to its leukemogenic effect. When p-glycoprotein is overexpressed (result of 3435CC genotype) both toxic and therapeutic compounds are quickly eliminated from the intracellular space decreasing their effect and therapeutic mechanism. We were also capable of defining new predictive models using genetic variables along with clinical ones.

NOTA PRELIMINAR

LISTA DE PUBLICAÇÕES

ANEXO 1: **"Impact of therapy related AML in the outcome of Portuguese Acute myeloid leukemia patients"**

Espirito Santo A., Chacim S., Ferreira I., Leite L., Moreira C., Pereira D., Dantas M., Nunes M., Viterbo L., Moreira I., Martins A., Oliveira I., Domingues N., Mariz J., Medeiros R.

Aceite para publicação em Janeiro 2015 na Oncology Letters

ANEXO 2: **"SWOG pretreatment risk criteria in Acute Myeloid Leukemia: predictive or prognostic factor?"**

Espirito Santo A., Chacim S., Ferreira I., Leite L., Moreira C., Pereira D., Dantas M., Nunes M., Viterbo L., Moreira I., Martins A., Oliveira I., Domingues N., Mariz J., Medeiros R.

Aceite para publicação em Dezembro de 2015 na Molecular and Clinical Oncology

ANEXO 3: **"Pharmacogenetic considerations for non-Hodgkin's lymphoma therapy."**

Espirito Santo A, Medeiros R.

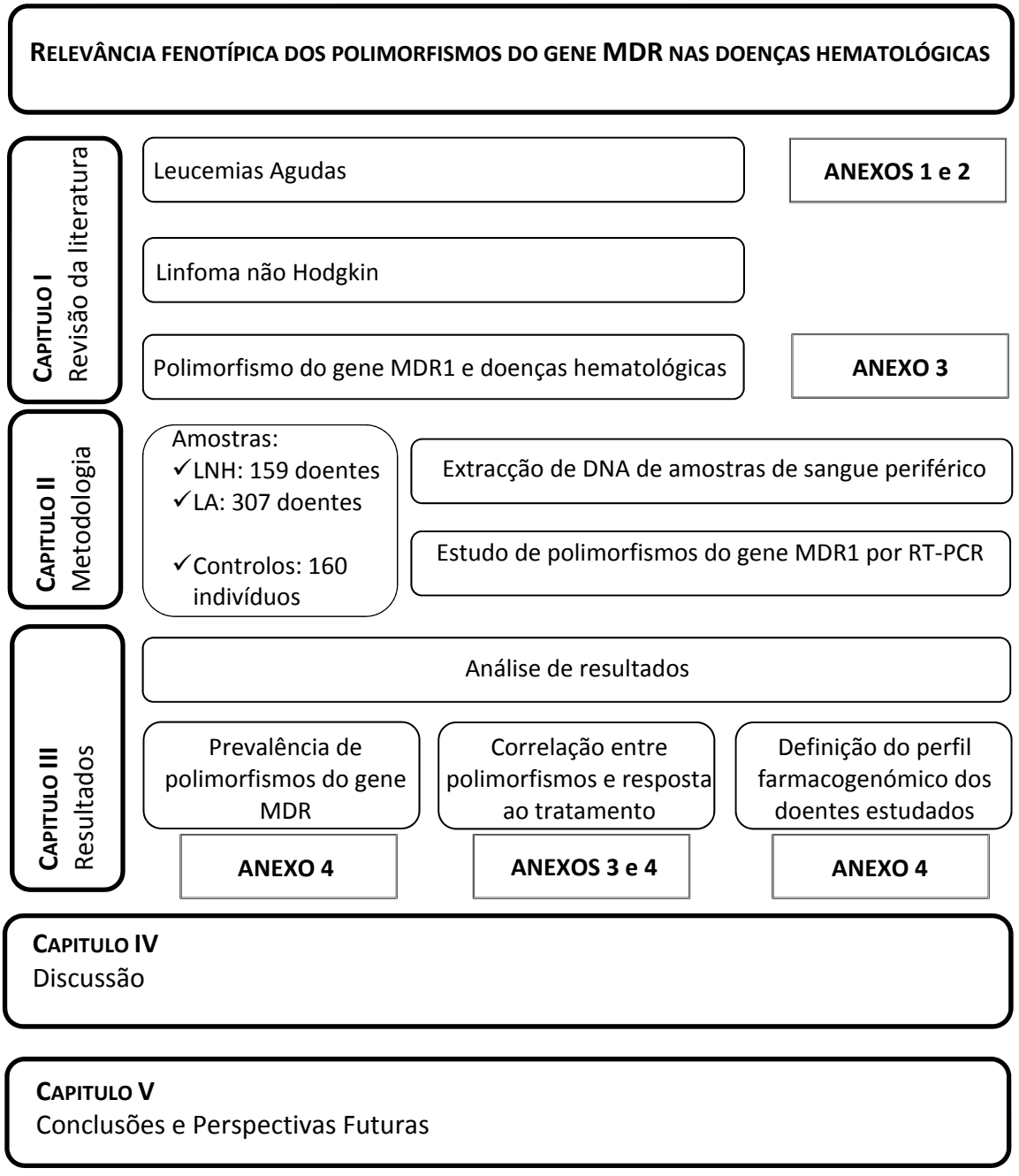
Expert Opinion on Drug Metabolism and Toxicology. 2013 Dec;9(12):1625-34. doi: 10.1517/17425255.2013.835803. Epub 2013 Sep 22

ANEXO 4: **"Multidrug Resistance Gene C1236T polymorphism influences risk for acute leukemia"**

Espirito Santo A., Bessa J., Gomes M., Mariz J., Medeiros, R.

Submetido para publicação

ORGANIZAÇAO DA TESE



1. REVISÃO DA LITERATURA

O uso de terapêutica citotóxica mantém-se a base do tratamento das doenças hematológicas malignas, nas modalidades terapêuticas atualmente disponíveis, com fins curativos ou apenas paliativos. A resistência primária à quimioterapia é a causa mais importante de morte relacionada com cancro. Esta situação advém de vários mecanismos quer do individuo quer da neoplasia, ou por uma combinação de factores que permitem à doença ultrapassar o efeito da terapêutica. Um destes mecanismos inclui a evolução clonal de linhas celulares intrinsecamente resistentes à terapêutica [2, 3]. As células malignas possuem resistência cruzada com vários compostos, a maioria de origem natural, que partilham entre si algumas características estruturais; são usualmente pequenas moléculas hidrofóbicas que entram no espaço intracelular por difusão passiva, atravessando a bicamada fosfolipídica, que podem ser substratos da P-gp para a saída do interior da célula para o espaço extracelular. A P-gp é uma bomba de efluxo, dependente do ATP. A alteração da expressão da P-gp é causa mais frequente de resistência primária a drogas.

As doenças hematológicas malignas incluem um grupo heterogéneo de doenças clonais com origem na célula progenitora hematopoiética. Apesar dos resultados publicados relativos à influência dos polimorfismos do gene MDR1, e da consequente expressão da P-gp, serem por vezes controversos, existe uma tendência na demostração da relação entre as doenças hematológicas malignas e o gene MDR1. O efeito dos polimorfismos do gene MDR1 e da expressão da P-gp relacionam-se com risco para o desenvolvimento da doença maligna pela perpetuação da agressão tóxica

à célula, com a sobrevida pela diminuição do efeito dos fármacos pela sua excreção mais rápida, mas também com o aumento da toxicidade dos tratamentos decorrente da acumulação excessiva de compostos no espaço intracelular.

LEUCEMIA AGUDA

"Leucemia aguda" (LA) define um grupo heterogéneo de doenças neoplásicas, com origem na célula hematopoiética progenitora, que mantém a capacidade de autorrenovação [4].

As células neoplásicas podem ter filiação mielóide no caso da Leucemia mielóide aguda (LMA) ou linfóide na Leucemia linfóide aguda (LLA). A linhagem da LA tem implicações quer na terapêutica quer no prognóstico.

Até 2002 a classificação das LA baseava-se unicamente nos achados morfológicos (complementados pela citoquímica). Em 2002 e em 2008 [5, 6] a OMS definiu uma nova classificação que inclui, para além das características morfológicas, as características biológicas (alterações citogenéticas e moleculares) da doença. O conhecimento mais profundo da leucemogénese permitiu o desenvolvimento de novas armas terapêuticas dirigidas a alvos genéticos específicos.

DIAGNÓSTICO

O diagnóstico baseia-se na observação do aspirado de medula óssea. Os achados morfológicos são assessorados pela análise por técnicas como imunofenotipagem, citogenética e biologia molecular [5-7].

Para o diagnóstico de leucemia aguda é necessário a presença de pelo menos 20% de blastos na medula óssea.

São exceções a esta regra: a presença da t(8,21)(q22;q22), inv16(p13.1;q22) ou t(16,16)(p13.1;q22) ou t(15,17)(q22;q12), a Leucemia eritróide pura em que para o diagnostico é necessária a presença de mais de 80% de percursores eritróides, a Leucemia mielomonocítica e megacarioblástica em que a percentagem de blastos é substituída pela presença de monoblastos ou promonócitos e megacarioblastos respectivamente e os casos de proliferação extramedular de células hematopoiéticas imaturas (sarcoma mielóide), que são suficientes para o diagnóstico de LMA [5-7].

Na classificação da OMS de 2008 LLA e Linfoma linfoblástico são a mesma entidade distinguida apenas pela localização de doença predominantemente na medula óssea no caso da LLA ou nodal e extranodal no caso do Linfoma linfoblástico [7]

INCIDÊNCIA

Na LLA a incidência ajustada à idade é de 1,5 casos / 100000 habitantes. Dos 4000 novos casos diagnosticados por ano nos EUA a maioria encontra-se abaixo dos 15 anos de idade; a mediana de idade ao diagnóstico é de 13 anos. A distribuição etária é

bimodal com o 1º pico verificando-se entre os 2 e os 5 anos de vida e o 2º após os 50 anos. A incidência é maior na raça negra e nos homens (ratio H:M de 1,2:1) [8-11].

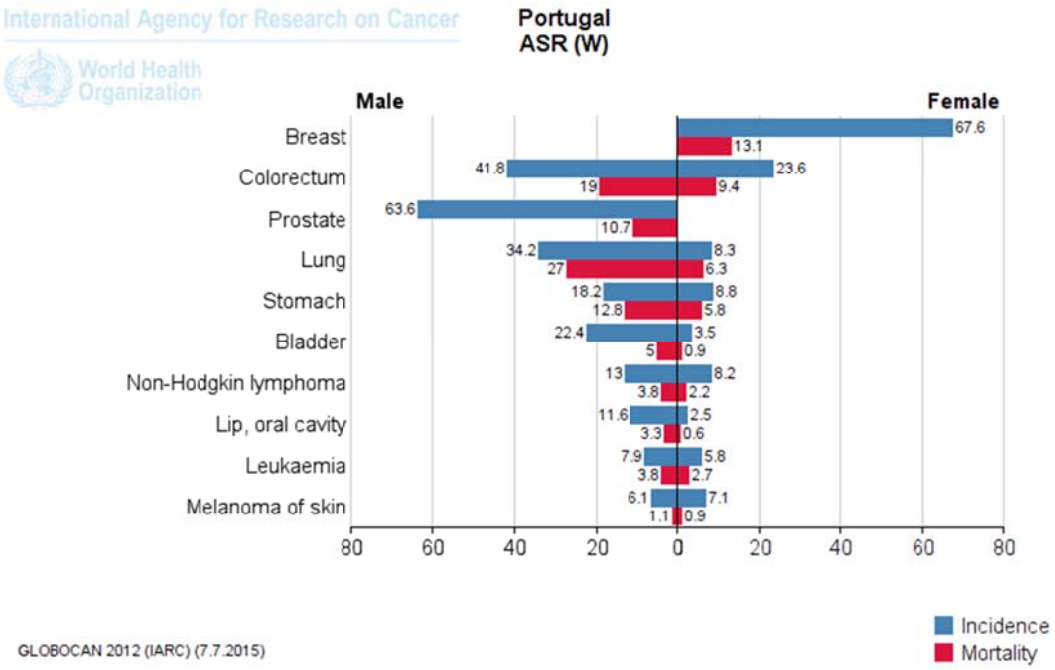


Figura 1.1: Incidência e mortalidade das 10 neoplasias mais frequentes em Portugal

Ao contrário da LLA, a LMA é a Leucemia Aguda do adulto [10, 11]. A idade média ao diagnóstico é de 67 anos. A sua incidência aumenta com a idade sendo de 1 / 100 000 habitantes abaixo dos 30 anos de idade e de cerca de 17 / 100 000 habitantes acima dos 75 anos de idade. Representa cerca de 15 % dos casos de LA em idades inferiores a 10 anos e 80-90 % na idade adulta. É mais frequente nos homens e nos caucasianos. A sua incidência é também maior nos países desenvolvidos e nas cidades industrializadas [8, 12, 13].

Em Portugal as Leucemias encontram-se na lista das 10 neoplasias mais frequentes (figura 1.1).

FACTORES DE RISCO

Factores genéticos [14, 15] foram implicados na patogénese da LMA pela sua alta incidência em doentes com síndromes associados a defeitos cromossómicos (como o síndrome de Down, síndrome de Bloom, síndrome da monossomia 7, síndrome de Kleinefelter, síndrome de Turner, neurofibromatose) ou instabilidade do ADN como os síndromes de falência medular congénita (anemia de Fanconi, disqueratose congénita, síndrome de Schwachman-Diamond, trombocitopenia amegacariocítica, agranulocitose de Kostmann, anemia aplásica familiar, distúrbio familiar das plaquetas). O estudo da patogénese destas doenças, particularmente dos síndromes de falência medular congénita, levaram ao aprofundar do conhecimento do processo de leucemogénese [16].

Os factores hereditários e os síndromes de predisposição genética acima enumerados relacionam-se também com a LAL das crianças. A causa etiológica para o aparecimento desta forma de leucemia aguda em adultos grandemente desconhecida [17].

A radiação ionizante [18-21], os compostos aromáticos derivados do benzeno [22-25] e os pesticidas [19, 26, 27] são considerados tóxicos com potencial leucemogénico e implicações em LAM e LAL. A exposição ocupacional a baixa dose de radiação ionizante está associada à LLA do adulto enquanto as altas doses de Hiroshima e Nagasaki aumentaram a incidência em crianças [18].

FACTORES DE PROGNÓSTICO

A estratificação de risco é efetuada ao diagnóstico e tem implicações terapêuticas.

O imunofenótipo (linhagem B ou T, madura ou percursora), a idade [28, 29], a existência de hiperleucocitose [30], as alterações citogenéticas envolvendo o gene MLL [31-33] e o gene de fusão BCR-ABL [34, 35] são factores de prognóstico na Leucemia linfóide aguda.

Tabela 1.1: Factores de prognóstico da LMA definida pelo grupo SWOG

	Citogenética	**Alterações moleculares**
Favorável	"Core binding factor" (inv 16, t(16,16), t(8,21)) t(15,17)	Citogenética normal com mutação NMP1 na ausência de FLT3-ITD ou mutação bialélica isolada CEBPA
Intermédio	Normal +8 isolada t(9,11)	t(8,21), inv 16 ou t(16,16) com mutação c-Kit
Desfavorável	Complexo (≤ 3 alterações) Cariótipo monossómico -5, 5q-, -7, 7q- 11q23 - excepto t(9,11) inv 3, t(3,3) t (6,9) t (9,22)	Citogenética normal com mutação FLT3-ITD

Na Leucemia mielóide aguda o prognóstico envolve a idade, as alterações citogenéticas ou moleculares mas também se relaciona com a existência de uma neoplasia prévia (hematológica ou não) [36-38]. Por esse motivo a classificação da OMS de 2002 [5], revista e atualizada em 2008 [7], contempla as alterações

citogenéticas e moleculares mas também a relação da LMA com o tratamento efectuado para neoplasia prévia.

Alguns grupos cooperativos internacionais definiram escalas de prognóstico. Na tabela 1.1 está presente a estratificação do grupo SWOG [39, 40].

TRATAMENTO

O tratamento das leucemias agudas é modular, ou seja, é constituído por várias fases sequenciais, em que o início da seguinte depende do resultado da anterior.

A primeira fase é a Indução. O objetivo desta terapêutica inicial, de apenas um ciclo, é alcançar resposta completa. Considera-se resposta completa quando na medula óssea se observam menos de 5% de blastos (na morfologia) e há recuperação hematológica (glóbulos brancos superiores a 1 000 $x10^6$ /mm^3 e plaquetas superiores a 100 000 /mm^3) [7].

Após alcançar este objetivo segue-se a consolidação. Esta fase compreende vários ciclos de quimioterapia, iguais ou diferentes entre si, com o objetivo de reduzir ao máximo a doença residual.

A fase seguinte de manutenção realiza-se apenas nas Leucemias linfoblásticas agudas e na Leucemia mielóide aguda com a t(15,17) (Leucemia aguda promielocítica).

O tratamento profilático da infiltração do sistema nervoso central e a realização de Alotransplante de medula óssea poderão também fazer parte do tratamento, quando recomendado.

Igualmente importante é o tratamento de suporte. Deste fazem parte as condições de isolamento dos doentes, a profilaxia infeciosa, o tratamento agressivo e atempado das infeções e o suporte transfusional. Assim, nas últimas décadas, temos evoluído na profilaxia das infeções muitas vezes fatais para estes doentes, com melhor isolamento dependente de medidas de proteção mais eficazes com o uso de ar filtrado e de quartos com pressão positiva [41] mas também do reforçar institucional da assépsia das mãos. O uso de acessos venosos centrais mais eficientes, de hemoderivados mais seguros, de antibióticos mais potentes e eficazes e de unidades de tratamento mais diferenciadas contribuíram para o melhor tratamento e o aumento de sobrevida destes doentes.

O tratamento da LMA baseia-se na associação de citarabina com antraciclina [42-44]. A citarabina atua na fase S do ciclo celular, enquanto as antraciclinas atuam ao longo de todo o ciclo, criando sinergismo entre as duas classes de drogas.

Múltiplos esquemas de quimioterapia foram testados ao longo do tempo, com doses diferentes de citarabina, em perfusão ou em bolús, mas também com diferentes antraciclinas (idarrubicina, doxorrubicina, daunorrubicina) em diferentes doses e formas de infusão [45-49].

A substituição da antraciclina por mitoxantrone foi também avaliada estando recomendado o seu uso em casos específicos como nos idosos e nas recaídas [50].

A associação de etoposideo ao protocolo padrão de citarabina e antraciclina (“7+3”) foi também avaliado não se tendo verificado beneficio [51].

Devido à importância dada à P-gp e à sua expressão na refratoriedade da LMA à terapêutica, alguns agentes moduladores da actividade da P-gp foram associados à

quimioterapia [52-56]. O fármaco mais estudado e com relatos de algum potencial benefício foi a ciclosporina A [57-61].

Como estratégia de consolidação a citarabina em altas doses atingiu elevadas taxas de resposta, mas com potencial tóxico relevante. Assim outros esquemas de citarabina em doses menos intensas e em diferentes tipos de infusões foram testados [62-65].

Os análogos das purinas foram também utilizados em associação com a citarabina uma vez que a fludarabina administrada antes da citarabina aumenta a sensibilidade das células a esta droga, sendo também relevante nos casos de aumento de expressão da P-gp [66-70].

O tratamento dos doentes adultos com Leucemia Linfóide aguda baseia-se na poliquimioterapia com vincristina, corticoterapia e antraciclina. Estes protocolos mimetizam os protocolos pediátricos que alcançaram grande sucesso. A vantagem destes esquemas reside no uso de grandes doses cumulativas de drogas como corticoesteróides, vincristina e L-asparaginase assim como no tratamento mais precoce, regular e intenso do envolvimento do sistema nervoso central.

Na população de adultos jovens (idades entre os 15 e os 21 anos) devem ser utilizados esquemas de tratamento pediátricos, com vantagem na sobrevida (sobrevida aos 5 e 7 anos de 74 e 67%). Os protocolos pediátricos usam uma grande variabilidade de fármacos, administrados de uma forma muito intensiva, quer em dose quer em frequência, e com grande focalização no tratamento do sistema nervoso central [71-75].

O tratamento dos doentes adultos alicerça-se também na variedade de fármacos usados. Esquemas intensivos como o HyperCVAD (ciclofosfamida, vincristina, doxorrubicina, dexametasona, metotrexato, citarabina) são amplamente utilizados com sobrevida aos 5 anos de 42% [76, 77] . O grupo CALGB tem também uma estratégia de tratamento semelhante definida para esta patologia que atinge uma sobrevida aos 3 anos de 62% [78].

A estes esquemas pode ser associado o rituximab se as células leucémicas expressarem CD20 à sua superfície. Nesta situação as taxas de sobrevida sobem de 47 para 75% [79, 80].

O uso do Alotransplante de medula óssea como estratégia de consolidação destina-se a casos de alto risco (definido com hiperleucocitose, alterações citogenéticas associadas a mau prognostico – hipodiploidia e rearranjos envolvendo o MLL) [81-84].

A eficácia do tratamento dos doentes adultos com Leucemia Linfóide aguda Filadélfia positiva é extremamente baixa. Antes do uso dos inibidores da tirosina-cinase (iTC) a sobrevida aos 3 anos era de 20% [85]. Com a associação do imatinib à quimioterapia padrão verificou-se um aumento substancial na sobrevida [85-87]. A eficácia desta combinação é reforçada pelo uso do Alotransplante de medula óssea como estratégia de consolidação. Variadíssimos estudos efetuados por múltiplos grupos de estudo internacionais mostraram exatamente a importância do uso de iTC associados a quimioterapia e da realização subsequente de Alotransplante de medula óssea [80, 87-89].

SOBREVIDA

No caso dos doentes com Leucemia mielóide aguda cerca de 60 a 80 % irão atingir a resposta completa com o ciclo de indução [90-92].

A sobrevida destes doentes varia fundamentalmente com o risco citogenético, assim o risco favorável tem uma sobrevida estimada aos 5 anos de 70%, o intermedio de 48 % e o desfavorável de 15% [40].

As taxas de cura e sobrevida dos doentes com Leucemia Linfóide aguda melhoraram imenso nas últimas décadas, principalmente na população pediátrica [93, 94]. Esta melhoria da sobrevida deve-se fundamentalmente ao aumento do conhecimento dos mecanismos de doença, da sua genética e etiopatogenia, do tratamento adaptado ao risco e da terapêutica alvo.

Dados publicados mostram a sobrevida substancialmente maior em crianças (sobrevida aos 5 anos entre 86-89%) mas em decréscimo progressivo com o envelhecimento (adultos jovens 42-63%, adultos 24,1% e acima dos 60 anos de idade 17,7%) [28, 95, 96].

LINFOMA NÃO HODGKIN

DIAGNÓSTICO

Os linfomas são um grupo heterogéneo de doenças hematológicas malignas com origem nas células hematopoiéticas do tecido linfóide, correspondendo a cerca de 3% das neoplasias malignas do mundo inteiro [97].

O seu diagnóstico é fundamentalmente histológico. As técnicas atuais de diagnóstico incluem não só a histologia apoiada na imunocitoquimica mas também imunofenotipagem e o estudo genético.

INCIDÊNCIA

A incidência de LNH nos EUA em 2005 foi de 19,5 casos / 100 000 habitantes. Mais de 60 000 novos casos por ano são esperados na mesma população [97].

É mais frequente em indivíduos adultos do género masculino. A idade média ao diagnóstico situa-se entre os 45 e os 55 anos [97].

Verificou-se recentemente um aumento da incidência de LNH que se deve ao desenvolvimento deste tipo de patologia nos doentes com síndrome de imunodeficiência humana adquirida mas também ao aumento na eficácia e melhoria das técnicas de diagnóstico [98].

A forma de apresentação mais frequente envolve os gânglios (nodal), tendo na maioria dos linfomas origem na célula B. A apresentação extranodal corresponde a cerca de 15-25% dos casos da Europa e EUA, mas cerca de 50% na Ásia [99]. Os locais

extranodais mais frequentes são o trato gastrointestinal, nasofaringe, cérebro, pele, osso, tiroide, glândulas salivares e testículo.

FACTORES DE RISCO

Múltiplas infeções foram associadas ao aumento de risco de LNH, o que explica as variações de incidência geográficas [100-103]. Há pelo menos 2 mecanismos de linfomagénese descritos. O primeiro está associado à transformação direta do linfócito pelos agentes microbianos, como nos vírus linfotrópicos EBV, HTLV1 e HHV8. Outro mecanismo é o que se observa na infecção por HP que infecta os tecidos levando a uma resposta linfoproliferativa que se mantém dependente da presença do microrganismo. O tratamento neste último caso inclui antibióticos para erradicação da bactéria e desaparecimento do LNH.

Múltiplos químicos, quer de uso terapêutico quer de exposição ocupacional, foram também associados a aumento de risco de LNH [104-108].

Nos estados de imunodepressão (congénita ou adquirida) há desregulação do sistema imune, levando à diminuição da produção das citoquinas, originando a produção descontrolada de células B no tecido linfóide. Esta hiperplasia linfóide está frequentemente associada a infecção por EBV e pode ocorrer nos doentes transplantados recetores de órgãos sólidos em terapêutica imunossupressora [109, 110].

Nos doentes com SIDA os linfomas são fundamentalmente de células B, extranodais. Há inclusivamente formas de LNH exclusivas de doentes com SIDA, como

são os casos do Linfoma plasmablástico da cavidade oral e o linfoma primário das cavidades [111]. Com a instituição da terapêutica HAART ("highly active antiretroviral therapy" – terapêutica antirretroviral altamente ativa) a incidência dos LNH parece ter diminuído mas a percentagem de casos em que o diagnóstico de LNH levou à definição de SIDA aumentou [111-113].

Outras situações são também consideradas como influenciadoras do risco de LNH, como os casos da inflamação crónica, a hiper-reactividade imune e a imunossupressão características das doenças autoimunes [114]. São mais frequentemente extranodais, localizados e envolvendo células B, de baixo grau, tipo MALT.

FACTORES DE PROGNÓSTICO

Em 1971[115] um grupo cooperativo de estadiamento de Linfoma de Hodgkin actualizou o estadiamento desenvolvido inicialmente em 1959 em que considerava para o estadiamento apenas as áreas anatómicas. O novo estadiamento, conhecido com estadiamento de Ann Arbor, incluiu não só as áreas envolvidas mas também a sintomatologia sistémica do doente. Este sistema foi melhorado ao longo dos anos [116] mantendo relevância, sendo ainda usado para o estadiamento do LNH. Atualmente existem também escalas de prognóstico definidas para alguns subtipos de linfoma que usam o estadiamento de Ann Arbor como critério. Assim para os Linfomas agressivos existe o International Prognostic Index (IPI) [117], para o Linfoma de células do manto existe o MIPI (Mantle cell Lymphoma International Prognostic Index) [118] e

o FLIPI (Follicular Lymphoma International Prognostic Index) para o Linfoma de centro folicular [119].

TRATAMENTO

A estratégia terapêutica varia de acordo com o tipo de linfoma e com o estadiamento da doença. O tratamento dos LNH de uma forma geral baseia-se no protocolo CHOP [120]. A este esquema podem ser associadas outras drogas necessárias em situações especiais como o rituximab nos casos de LNH CD20+ [121-123].

Nos linfomas de baixo grau a primeira abordagem pode ser de vigilância. Como opções terapêuticas em 1ª linha temos a quimioterapia convencional com esquema CHOP ou semelhante, radioterapia de baixa dose [124, 125]ou rituximab em monoterapia [126].

Os LNH de alto grau exigem terapêuticas de intenção curativa mais agressivas. Assim nos LNH DGCB preconiza-se a terapêutica com o esquema RCHOP (ou semelhante), nos LNH de Burkitt é necessário tratamento de quimioterapia mais agressivo e intensivo. Esquemas mais elaborados como o BFM95 são muitas vezes utilizados com boas taxas de resposta [79, 127, 128]. Os LNH linfoblásticos são tratados com esquemas idênticos aos das LLA [129, 130]

A profilaxia ou tratamento do envolvimento do SNC poderá estar indicado em alguns caso necessitando do uso de metotrexato em altas doses em perfusão endovenosa [131-134].

O autotransplante de medula óssea como estratégia de consolidação em LNH pode ser uma opção terapêutica [135].

SOBREVIDA

Pela imensa heterogeneidade deste grupo que condiciona uma diversidade extrema de protocolos de quimioterapia a sobrevida é também muito diversa, ou seja, temos doenças curáveis em percentagem muito elevada (como os Linfomas de Burkitt) e outras que se comportam como doenças crónicas, recidivantes e incuráveis (como os linfomas de baixo grau).

Os doentes com LNH de Burkitt têm a melhor sobrevida global que atinge cerca de 90 % nos respondedores à terapêutica de 1ª linha; esta taxa diminui para menos de 50% quando os doentes ou são refratários ou recaiem. Nos Linfomas difusos de grandes células B a sobrevida esperada varia entre 87 e 44% de acordo com os grupos de prognóstico definidos pelo IPI [117]. No Linfoma de centro Folicular usando também a definição do FLIPI de grupos de prognóstico a sobrevida aos 10 anos varia de 71 a 36% [136].

O LNH de células do manto apresenta uma mediana de sobrevida aos 5 anos de cerca de 60 % nos doentes de baixo risco (MIPI) [118].

POLIMORFISMOS DO GENE MDR

GENE MDR1 E P-GLICOPROTEÍNA

A descrição inicial do gene MDR1 data de 1973, tendo sido identificado nas células ováricas de hamsters chineses. Este gene localiza-se no braço longo do cromossoma 7 (localização 7q21.12) [137-139]. Codifica uma proteína chamada P-glicoproteína (figura 1.2) com um peso molecular de 170 KDa.

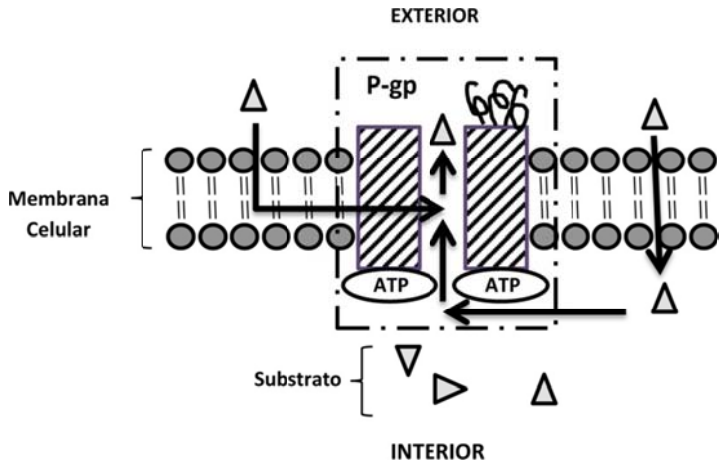


Figura 1.2: Representação esquemática da P-glicoproteína

Esta proteína é uma bomba de efluxo relacionada com a resistência primária a fármacos. É composta por duas metades homólogas, provavelmente originadas por duplicação génica. Cada uma destas metades tem 6 domínios extracelulares, um local de ligação dependente do ATP, dois motivos (Walker A e B) e locais glicosilados no exterior (figura 1.2). A fosforilação desta proteína é fundamental para a sua actividade

de exportação de substâncias do interior. Os substratos desta proteína são moléculas hidrofóbicas e anfipáticas capazes de entrar na célula por difusão passiva. A ligação entre a P-gp e o substrato ocorre ao nível intracelular ou na dupla camada fosfolipídica, uma vez que a função primordial desta proteína é de transportar ativamente o substrato do espaço intra para o extracelular.

A P-gp encontra-se em quase todos os tecidos independentemente da sua função. No entanto o nível de expressão será maior nos tecidos epiteliais que têm funções de excreção, como o revestimento epitelial do colon, intestino delgado, ductos pancreáticos e biliares, sistema excretor renal e glândulas suprarrenais [139-142]. A P-gp está também presente nas células endoteliais das barreias sanguíneas existentes no cérebro, testículo, tecido mamário e ouvido interno [143]. A sua expressão é também elevada na placenta e no endométrio das mulheres grávidas. Em todos os locais em que está presente esta proteína assume um papel protetor.

Polimorfismos (SNP) são as variações de sequência mais frequentes de um gene. Mais de 50 SNP foram identificados no gene MDR1 [144-147]. Há no entanto 3 - C3435T, C1236T e C2677T/A, considerados mais frequentes (cerca de 10%) em variadas populações. Estes 3 polimorfismos estão geneticamente ligados, levando à formação dos 2 haplótipos mais comuns (1236C-2677G-3435C e 1236T-2677T-3435T) que se pensa estarem relacionados com diferentes mecanismos de alteração da expressão e da função da P-gp. A diminuição do transporte do substrato por esta bomba extrusora pode estar relacionada com a existência de uma estrutura transportadora disfuncionante incapaz de se ligar ao substrato. Os SNP alteram a

sequência génica levando à formação de uma proteína com diminuição da capacidade de ligação [144, 148-154].

POLIMORFISMOS DO GENE MDR1 E DOENÇAS HEMATOLÓGICAS

Os resultados publicados sobre a influência dos polimorfismos do MDR1 nas LA são pouco concordantes. No caso da Leucemias agudas linfoblásticas [149-151], especialmente os estudos em crianças, foi demonstrada a relação dos polimorfismos do gene MDR1 com o aumento da susceptibilidade ao efeito leucemogénico dos pesticidas [155]. Nas LMA não se encontram dados na literatura que relacionem os polimorfismos do MDR1 com risco [156-160]. Estes mesmos polimorfismos parecem não influenciar a sobrevida em LMA e em LLA (tabela 1.2) [156-160].

Não existe relação documentada na literatura até à presente data entre polimorfismos do gene MDR1 e risco para LNH (tabela 1.2)[161].

Nos estudos desenvolvidos verificou-se a associação entre o polimorfismo G2677T/A e a evolução clinica, quer como fator independente quer em associação com o polimorfismo C3435T. Para além disso o estado de portador do alelo T neste mesmo local foi associado à diminuição da sobrevida e à resistência a drogas [162]. O polimorfismo C3435T foi relacionado com a eficácia da terapêutica [161].

Não foi observada relação entre o polimorfismo C1236T e sobrevida de LNH[163].

Tabela 1.2: Influência em risco e sobrevida dos polimorfismos do Gene MDR1 em LLLA, LMA e LNH

Polimorfismo	Efeito	LLA	LMA	LNH
C3435T	**Risco**	Susceptibilidade genética [164-166] Genótipo TT influencia risco [167, 168] Influencia na susceptibilidade do efeito leucemogénico dos pesticidas [155]	Reduz o risco de progressão de SMD para LA pelo aumento da apoptose [169]	Não influencia risco para LNH [161]
	Sobrevida	Genótipo CC associado a pior prognóstico [167, 168] Não explica por si só a variabilidade na farmacocinética da vincristina [170] Não influencia a resistência a drogas[171]	Sem implicações clinicas [156-160] Influência de variantes alélicas[172]	Determina a eficácia da quimioterapia [161] Efeito combinado com o polimorfismo G2677T/A na sobrevida global de doentes com LNH difuso de grandes células B [162]
C1236T	**Risco**	Influencia na susceptibilidade do efeito leucemogénico dos pesticidas [155]		
	Sobrevida	Biomarcadores potenciais na sobrevida e prognóstico [173]	Não significativo [157] Influência de variantes alélicas[172]	Não influencia a resistência a drogas [163]
G2677T/A	**Risco**	Influencia na susceptibilidade do efeito leucemogénico dos pesticidas [155]		Não influencia risco para LNH [161]
	Sobrevida	Diferenças significativas encontrada Não explica por si só a variabilidade na farmacocinética da vincristina [170]	Pode predizer a resposta ao tratamento de indução [174] Sem significado [157, 158, 160] Influência de variantes alélicas [172]	Fator de prognóstico independente para sobrevida global [162] Genótipo T associado com menor sobrevida e maior resistência a drogas [162] Efeito combinado com o polimorfismo C3435T na sobrevida global do LNH difuso de grandes células B [162]

EXPRESSÃO DA P-GLICOPROTEÍNA E DOENÇAS HEMATOLÓGICAS

A P-gp encontra-se expressa nas células blásticas de leucemia aguda quer ao diagnóstico quer após falência da terapêutica [175]. Esta expressão é maior em doentes resistentes quando comparados com doentes que responderam ao tratamento e nos idosos.

A sobrexpressão da P-gp é considerada a principal causa de resistência primária à terapêutica em doentes com LMA pois estudos demonstraram a correlação entre esta expressão aumentada e a resistência ao tratamento padrão com antraciclina e citarabina [176-178]. A ausência de resposta aliada ao aumento de expressão da P-gp pode justificar-se com um fenótipo mais agressivo da doença, mas também com o aumento da excreção do interior da célula dos fármacos citotóxicos .

A expressão da proteína foi relacionada com o polimorfismo do MDR1. Quando há homozigotia para o alelo T deste polimorfismo, há diminuição da expressão da proteína levando ao acumular de compostos no espaço intracelular. Estes agentes são tóxicos para a célula podendo aumentar o risco de leucemia aguda no caso de potenciais leucemogénicos ou aumentar os níveis da terapêutica para zonas tóxicas aumentando os seus efeitos laterais. Quando o alelo C está presente em homozigotia há aumento da produção da proteína transportadora o que origina o aumento da excreção dos xenobióticos do interior da célula, incluindo os agentes citotóxicos, diminuindo a sua eficácia e sobrevida da doença (figura 1.3) [151, 179, 180].

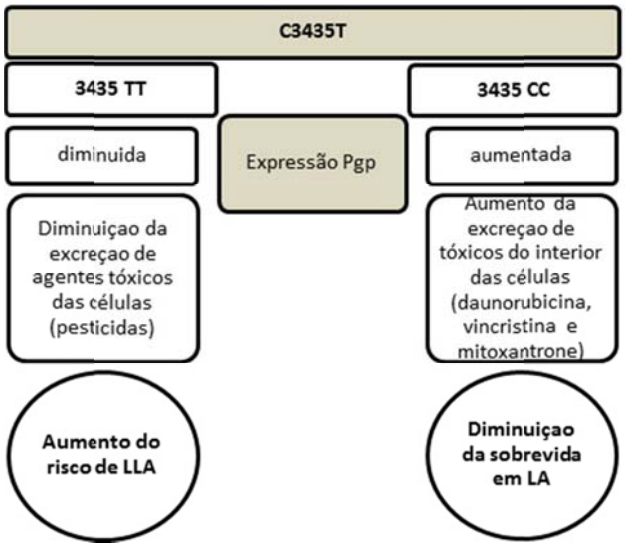


Figura 1.3: Representação esquemática da influência do polimorfismo C3435T na expressão de P-gp e na sua influência de risco e sobrevida em LA.

Estudos publicados demonstraram que a expressão de P-gp está alterada mesmo antes do diagnóstico de LNH. A associação entre a expressão de P-gp e o risco de desenvolvimento de LNH foi verificada quando foi estudado o tecido linfóide clonal e outras condições pré-malignas [181].

Em 1995 um estudo demonstrou a relação entre a expressão de P-gp e o desenvolvimento de LNH T [181]. Em 2001 e em 2007 foi também demostrado o aumento da expressão de P-gp em amostras de doentes com LNH de células B, quer ao previamente ao tratamento, quer na recaída [182, 183]. Em 2009 foi publicado um estudo que não encontra alterações na expressão da P-gp previamente ao tratamento [184].

A expressão de P-gp foi também associada com alguns tipos específicos de LNH. O grupo alemão de LNH cutâneo demostrou a relação entre a expressão de P-gp e o fenótipo de doença [185]. Também o linfoma NK/T nasal foi estudado por 2 diferentes grupos demonstrando-se em ambos o aumento da expressão de P-gp [183, 186].

Apesar da ambiguidade de resultados apresentados em que muitos falham em encontrar relação [187, 188], estudos mais recentes demonstram relação entre a expressão de P-gp e sobrevida de LNH. Esta associação foi demonstrada em situações de LNH DGCB, NK/T nasal, Burkitt e LNH relacionado com SIDA [182, 184, 189-193]. A associação com o tempo médio até progressão foi também definida [191].

REVERSÃO TERAPÊUTICA DA P-GLICOPROTEÍNA

As estratégias utilizadas para ultrapassar a resistência primária aos fármacos são variadas (tabela 1.3). Estas incluem a alteração da expressão da P-gp (como o benzufuran) [194], a remoção da proteína da bicamada fosfolipídica (como o rituximab) [195-197], a competição com o substrato (como os anti retrovíricos ritonavir e saquinavir) [198, 199], o aumento da apoptose das células com alta expressão de P-gp (como a ciclosporina e o valspodar)[57, 200, 201] ou o camuflar da droga com um componente não reconhecido pela P-gp como substrato (como a doxorrubicina lipossómica não peguilada) [202-204]. A representação esquemática destes mecanismos encontra-se na figura 1.4.

Tabela 1.3: Possíveis caminhos terapêuticos na reversão da resistência a drogas usados no LNH e LA

Intervenção terapêutica	Mecanismo de acção	Ref.
Benzofuran	Alguns dos 24 componentes mostraram boa actividade anti proliferativa e outros mostraram actividade reversora da actividade do MDR.	[194]
Inobuzumab	Efeito em uso combinado.	[205]
Doxorrubicina lipossómica não-peguilada	O encapsulamento lipídico pode ultrapassar a resistência induzida pelo MDR.	[202-204]
Rituximab e Anti CD19	O rituximab induz a P-gp a sair da camada lipídica. Pode quimissensibilizar as células P-gp (+) interferindo nas relações entre a P-gp e o CD19, resultando na translocação da proteína para a membrana plasmática onde não se encontra disponível para o substrato.	[197] [196]
Ritonavir e saquinavir	Podem tornar as células novamente sensíveis a fármacos como a vinblastina, vincristina e doxorubicina.	[199]
Bortezomib	Inibem potencialmente o crescimento e aumento a apoptose em linhas celulares. Bortezomib e daunorubicina têm efeito sinérgico na anti proliferação.	[206-209]
Valspodar	Análogo da ciclosporina não imunossupressor Inibidor potente P-gp Modula o metabolismo da ceramide importante na apoptose Pode potenciar a apoptose induzida por alguns fármacos	[53, 210]
Tamoxifeno	Aumenta a concentração de daunorrubicina intracelular inibindo o efluxo mediado pela P-gp Modula o metabolismo da ceramide importante na apoptose	[211]
Ciclosporina	Modula o metabolismo da ceramide importante na apoptose	[61, 212-214]
Perifosine	Inibidor do Akt Induz morte celular por apoptose, alterando a integridade da camada lipídica Diminui a expressão do ARN mensageiro da P-gp	[215-218]
Zosuquidar	Inibição da P-gp nos ductos biliares impedindo a excreção biliar	[55, 219-221]
Quinino	Reverte a actividade da P-gp	[222-227]
Ribossomas	Ribossomas de células *MDR 1* negativas cells são transfectadas em células que sobre expressam P-gp com reversão da expressão.	[228]

Apesar das variadas estratégias farmacológicas desenhadas com o intuito de ultrapassar este entrave genético à eficácia dos fármacos, só a ciclosporina tem resultados publicados de benefício no tempo até progressão com a sua adição aos esquemas de quimioterapia convencionais [50, 59-61].

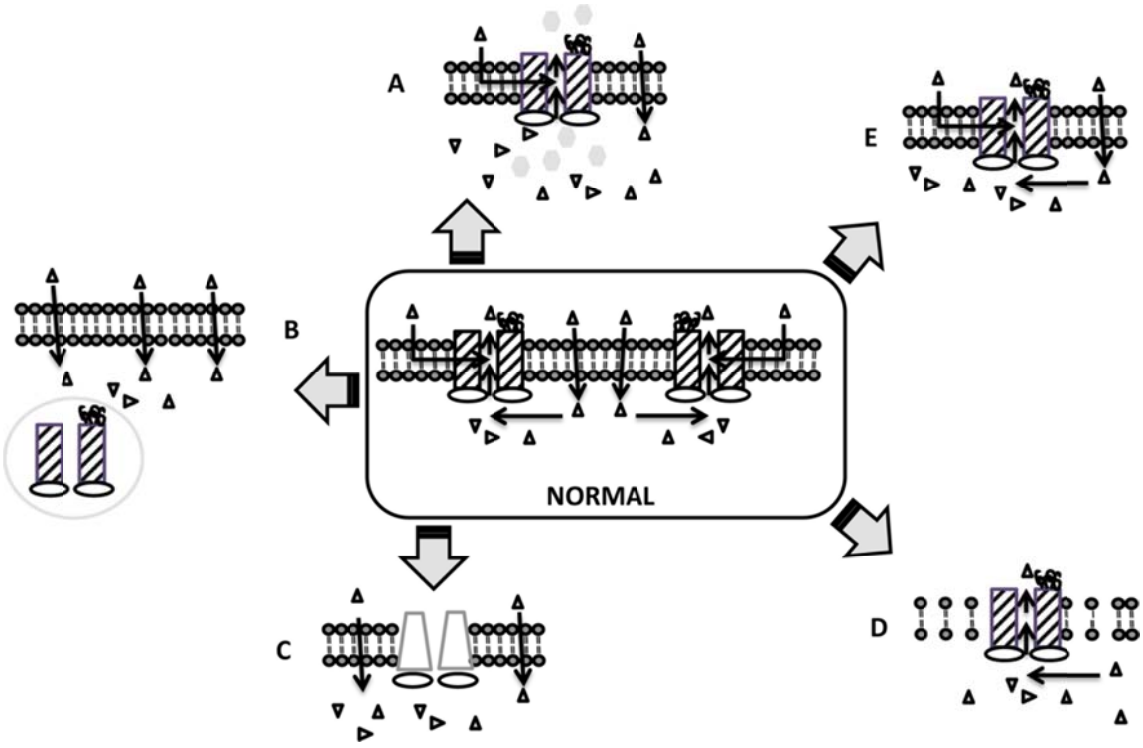


Figura 1.4: Representação esquemática dos mecanismos de modulação da P-gp.
A – inibição por outro substrato (ex: quinino), B – interiorização da proteína (ex: rituximab), C – alteração da proteína por SNP, D – Apoptose (ex: bortezomib), E – diminuição do RNAm com consequente diminuição da expressão da P-gp (ex: perifosine)

O principal obstáculo quando se ambiciona alterar a expressão da P-gp reside na possibilidade de alterar as normais funções celulares, uma vez que esta proteína está expressa em quase todos os tecidos. Por este motivo muitos fármacos foram

testados, mas o seu uso foi abandonado pela extrema toxicidade associada. A ciclosporina foi o primeiro fármaco usado capaz de atingir benefício sem grande aumento da toxicidade associada. A partir deste patamar mais 2 linhas de inibidores diferentes se desenvolveram: primeiro o valspodar [52, 53, 201, 229] que apresenta perfil de tolerância aceitável seguindo-se do zosuquidar [55, 56, 221] que é seletivo para a P-gp apresentando efeitos laterais mínimos.

A estratégia utilizada na doxorrubicina lipossómica não peguilada baseia-se na camuflagem. A molécula ativa apresenta-se envolvida numa camada lipídica que ultrapassa a ligação à P-gp logo não sendo exteriorizada da célula [202-204].

A eficácia do rituximab foi testada *in vitro* em linhas celulares resistentes [192]. Este anticorpo, conjuntamente com o anti CD19, alteram a localização da proteína na célula retirando-a da bicamada fosfolipídica inibindo a sua função [191].

No caso dos LNH associados a SIDA o uso dos anti retrovíricos como o ritonavir e saquinavir mostrou resultados na reversão da resistência primária uma vez que estas moléculas se ligam ao substrato inibindo o efluxo dos fármacos [199].

Outros fármacos há que induzem a morte celular das células com alta expressão de P-gp. São exemplos a ciclosporina, o valspodar, o tamoxifeno, o bortezomib e o perifosine [211, 215-218, 230-232].

O quinino, usado regularmente com outros fins terapêuticos, foi também associado a diminuição da expressão da P-gp [222-227].

2. OBJETIVOS

1. Prevalência de polimorfismos do gene MDR, dentro da população estudada e definição de um perfil farmacogenómico.

2. Avaliar a resposta ao tratamento, correlacionando estes dados com a existência de polimorfismos.

3. Definição do perfil farmacogenómico dos doentes estudados, elaborando um modelo de abordagem terapêutica direcionado.

3. METODOLOGIA E RESULTADOS

As metodologias utilizadas nos diferentes estudos realizados assim como a caracterização das amostras e do material são descritas em pormenor em cada artigo ou nas referências bibliográficas correspondentes.

Apresenta-se de uma forma resumida a descrição dos materiais e dos métodos utilizados na obtenção dos resultados apresentados nesta dissertação.

MATERIAL

O estudo é descritivo, prospectivo, não randomizado, não controlado. Inclui doentes com diagnóstico de Leucemia aguda (qualquer subtipo) e Linfoma não Hodgkin de células B (qualquer subtipo), selecionados a partir da base de dados do Serviço de OncoHematologia do Instituto Português de Oncologia do Porto (IPO-Porto) até Dezembro de 2010.

Foram incluídos 466 doentes, 307 com diagnóstico de Leucemia aguda e 159 com diagnóstico de Linfoma não Hodgkin de células B.

O trabalho apresentado foi aprovado pela Comissão de Ética do IPO-Porto. Todos os doentes selecionados assinaram o consentimento informado.

Critérios de inclusão:

- Todos os doentes têm diagnóstico efectuado, ou confirmado histologicamente, na instituição, constando estes dados no processo clínico.
- Sobrevivência previsível de pelo menos 1 mês à data de inclusão.
- Pacientes com idade igual ou superior a 18 anos.
- Decisão de tratamento com intenção curativa.
- Possibilidade de efetuar colheitas de sangue periférico (em veia periférica ou cateter venoso central).
- Consentimento informado assinado (considera-se necessário apenas o consentimento que consta no "Guia do utente" facultado aos doentes na primeira consulta).

Foram excluídos todos os doentes que não preencham os requisitos de inclusão.

Parâmetros avaliados:

- Características do doente: género, idade ao diagnóstico, data de diagnóstico, doenças concomitantes, exposição a tóxicos.
- Características da doença: diagnóstico, estadiamento (quando aplicável).
- Tratamento efectuado e resposta à terapêutica.

Colheita e gestão das amostras:

- As colheitas sanguíneas efetuadas em veia periférica ou cateter venoso central, em qualquer data do seu percurso terapêutico.
- Foi colhido sangue em tubo de EDTA (3 ml).
- A amostra de sangue total foi usada na extração de ADN para estudo de polimorfismos pela técnica de PCR.
- O remanescente foi congelado e armazenado para futuras confirmações de resultados por um período de 5 anos.

MÉTODOS DE ANÁLISE DE BIOLOGIA MOLECULAR

Amostras de sangue periférico em tubos contendo EDTA foram obtidas por venopunção. O ADN genómico foi extraído das amostras de sangue total usando um Kit comercial (E.Z.N.A. – Omega Bio-Teck, Norcross, USA) de acordo com as instruções do produtor e armazenadas a -20ºC.

Os polimorfismos do gene MDR1 C3435T and C1236T foram genotipados usando a técnica de Real Time PCR (RT-PCR) com o método de genotipagem de Single Nucleotide polymorphism Taqman® C___7586857_20 e C___7586662_10, respectivamente, produzidos pela Applied Biosystems® (figuras 2.1 e 2.2). Os resultados da genotipagem foram avaliados independentemente da evolução clinica. No caso de resultados dúbios a análise foi repetida.

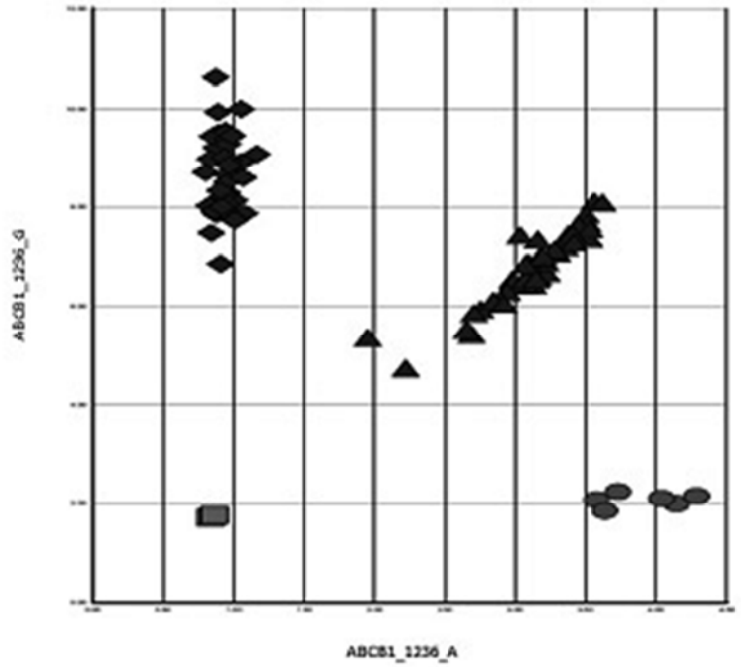


Figura 2.1: Análise do polimorfismo C1236T pela metodologia de discriminação alélica Taqman®

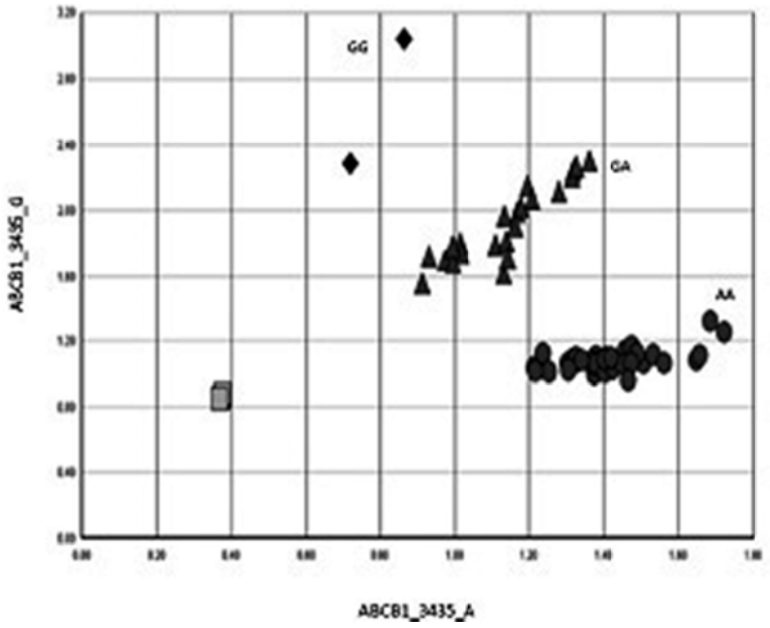


Figura 2.2: Análise do polimorfismo C3435T pela metodologia de discriminação alélica Taqman®

ANÁLISE ESTATÍSTICA

A análise dos dados foi efectuada utilizando o software Statistical Package for Social Sciences (SPSS version 17.0). A diferença dos resultados foi estimada usando o teste X^2. As probabilidades, medianas e as curvas de sobrevida foram definidas utilizando o método de Kaplan Meyer e analisadas utilizando o teste de Breslow (Wilcoxon), que é um teste de equivalência de distribuição de sobrevidas. Um valor de $p < 0,05$ foi considerado estatisticamente significativo.

A associação entre as frequências genotipicas do gene MDR1 e a incidência e prevalência das doenças estudadas (leucemia aguda e linfoma não Hodgkin) nas várias populações foi analisada utilizando o coeficiente de correlação de Pearson. A incidência e prevalência de leucemia aguda e linfoma não Hodgkin foi estabelecida a partir dos dados apresentados pela OMS (http://globocan.iarc.fr). A incidência dos genótipos dos polimorfismos C3435T and C1236T foram obtidos a partir dos dados publicados em http://www.appliedbiosystems.com.

4. RESULTADOS

LEUCEMIAS AGUDAS

Foram avaliados 307 doentes com diagnóstico de Leucemia aguda, diagnosticados e tratados no Serviço de OncoHematologia do IPO-Porto até 2010.

Na maioria destes doentes foi diagnosticada Leucemia mielóide aguda (n= 231; 75,3%). Os restantes 76 (24,7%) correspondiam a casos de Leucemia linfóide aguda. O resumo das características gerais destes doentes encontra-se na tabela 4.1.

Tabela 4.1: Caracterização da população

	LMA	LLA
Total doentes, n	231	76
Idade (mediana; anos)	50	37
Género feminino, n (%)	127 (55)	31 (40,8)
Prognóstico desfavorável	32 (17,3)	29 (38,7)
Resposta Completa, n (%)	150 (84,3)	53 (82,8)
AloTMO, n (%)	28 (12,1)	21 (27,6)
Recaída, n (%)	70 (46,7)	34 (56,7)
SG mediana (meses)	39	19
SG 5 anos (%)	45,9	28,6

LEUCEMIA MIELÓIDE AGUDA

São a maioria dos casos de leucemia aguda diagnosticada no adulto, refletindo-se também neste trabalho em que corresponde a mais de 75% dos casos estudados. A mediana de idade ao diagnóstico foi de 50 anos; predomina o género feminino (55% dos casos).

A maioria dos doentes tinha alterações no cariótipo inicial (n=128; 60,1%); os restantes 39,9% (n= 85) não tinham alterações citogenéticas. As alterações "core binding fator" foram as mais frequentemente identificadas, correspondendo a 56,3% de todas as cromossomopatias identificadas, seguidas pelas alterações complexas do cariotipo, demonstradas em 15,6% dos casos. As alterações numéricas (trissomias, monossomias, adições e deleções) corresponderam a 12,5% das situações. As restantes (15,6%) alterações encontradas eram aleatórias.

Estratificando os doentes de acordo com o esquema de prognóstico definido pelo grupo SWOG verificou-se que a maioria dos doentes se enquadrava no risco intermédio (n=107; 48,6%). No risco favorável encontravam-se 76 doentes (34,5%) e no risco desfavorável os restantes 37 doentes (16,8%).

A maioria (n= 137; 60,6%) foi tratada com esquema de quimioterapia contendo idarrubicina e citarabina ("7+3"). Trinta e oito doentes (16,8%) receberam tratamento de indução de acordo com o protocolo SWOG 9126 (antraciclina, citarabina e ciclosporina). O uso de esquemas de indução direcionados especificamente para a Leucemia aguda promielocitica (associada a t(15,17)) ficou limitado a este subgrupo de doentes, correspondendo a 43 casos (19%). Outros esquemas de tratamento de quimioterapia intensiva foram efetuados em 8 doentes (3,5%).

A grande maioria dos doentes obteve critérios de resposta completa após o tratamento de indução (n=176; 81,5%). Os restantes 40 doentes (18,5%) foram primariamente refratários.

Vinte e oito doentes foram submetidos a Alotransplante de medula óssea como estratégia de consolidação de resposta.

Dos doentes que obtiveram resposta a quimioterapia quase 40% recaíram (n= 70 doentes).

A sobrevida global dos doentes com LMA foi de 39 meses. A sobrevida aos 5 anos foi de 45,9%. O tempo médio até progressão foi de 15 meses (figura 4.1).

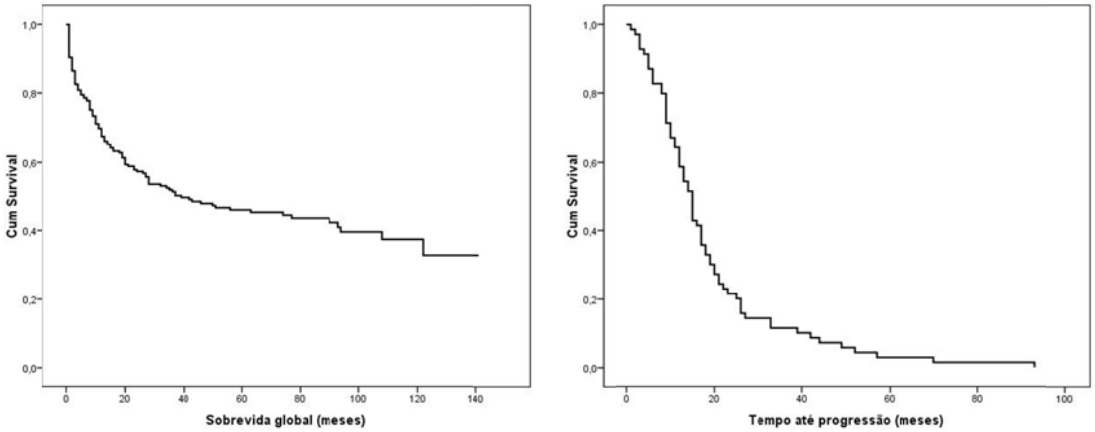


Figura 4.1: Gráfico da sobrevida global e do tempo até progressão nos doentes com LMA

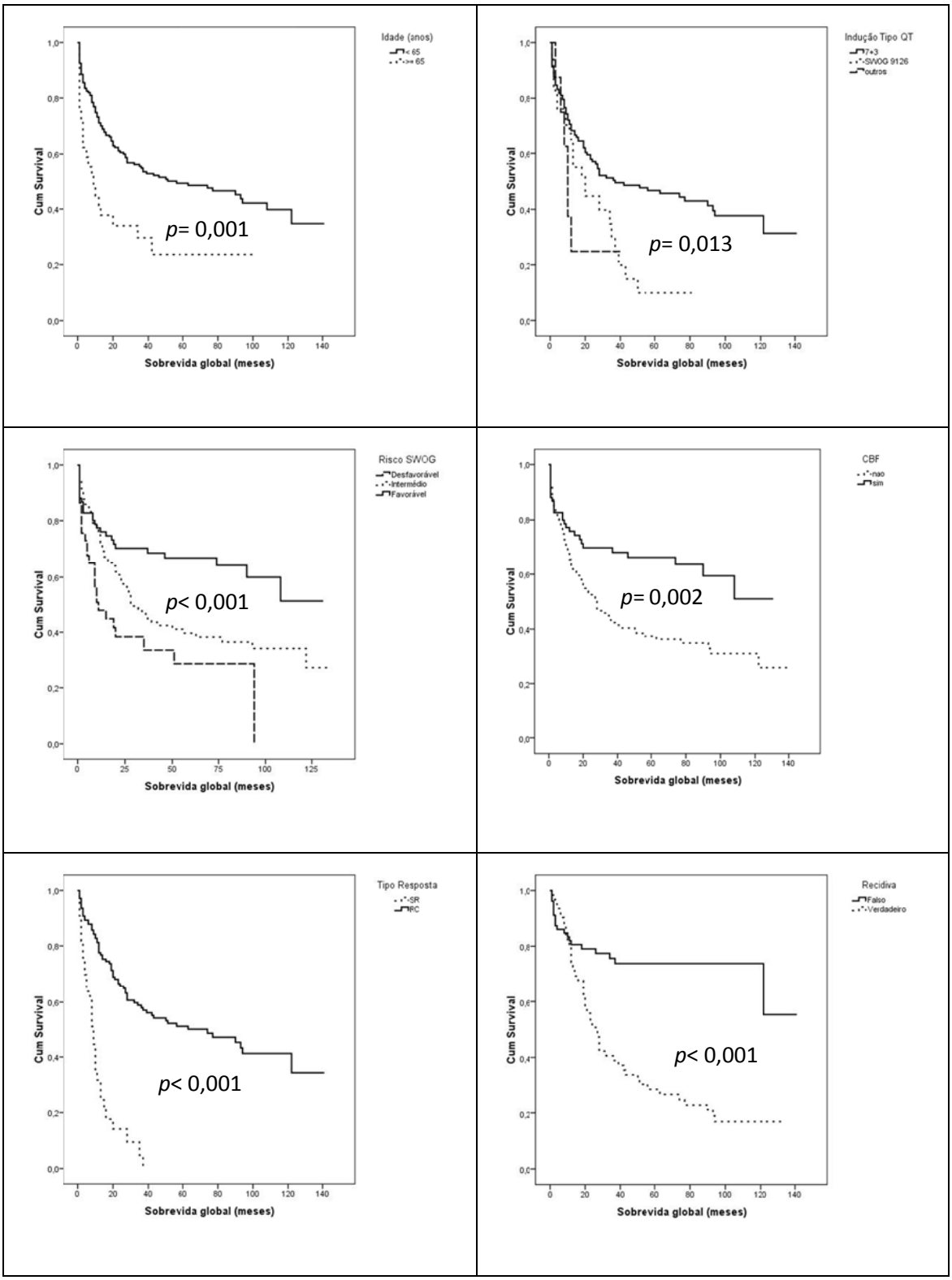


Figura 4.2: Gráficos ilustrativos da influência da idade, terapêutica instituída (excepto doentes com LMA promielocitica) risco citogenético, existência de anomalias CBF, tipo de resposta e recaída na sobrevida global dos doentes com LMA

Analisando separadamente os factores conhecidos condicionantes de resposta na nossa amostra podemos mostrar que a idade (p= 0,001), o risco citogenético (p< 0,001), a presença de anomalias CBF (p= 0,002), o protocolo de tratamento efectuado (p= 0,013), o tipo de resposta após a indução (p< 0,001) e a recidiva (p< 0,001) influenciam a sobrevida de uma forma estatisticamente significativa (figura 4.2).

Uma análise multivariada de regressão de Cox mostrou que há efeito de risco na sobrevida global da recaída (p= 0,001; 5,027 95% CI 2,908 – 8,689) e da adição de ciclosporina ao tratamento padrão (p= 0,030; 1,214 95% CI 1,019 – 1,446); observou-se efeito protetor na sobrevida global dos doentes estudados do risco citogenético favorável (p= 0,010; 0,610 95% CI 0,419 – 0,889) e da Leucemia aguda *de novo* (p= 0,001; 0,289 95% CI 0,140 – 0,597). A idade não mostrou influenciar a sobrevida global (p= 0,863; 0,931 95% CI 0,415 – 2,088) (tabela 4.2).

Tabela 4.2: Análise multivariada da sobrevida global

	p	**Hazard Ratio**
Idade	0,863	0,931 (95% CI 0,415 – 2,088)
Risco citogenético	0,010	0,610 (95% CI 0,419 – 0,889)
Tratamento de indução	0,030	1,214 (95% CI 1,019 – 1,446)
Recaída	< 0,001	5,027 (95% CI 2,908 – 8,689)
Leucemia *de novo*	0,001	0,289 (95% CI 0,140 – 0,597)

LEUCEMIA MIELÓIDE AGUDA RELACIONADA COM TERAPÊUTICA

Neste grupo de doentes identifica-se um subgrupo que corresponde aos doentes com diagnóstico de Leucemia aguda relacionada com terapêutica (Leucemia aguda secundária) com prognóstico mais desfavorável (sobrevida global de 10 meses; *p* <0,001) (figura 4.3).

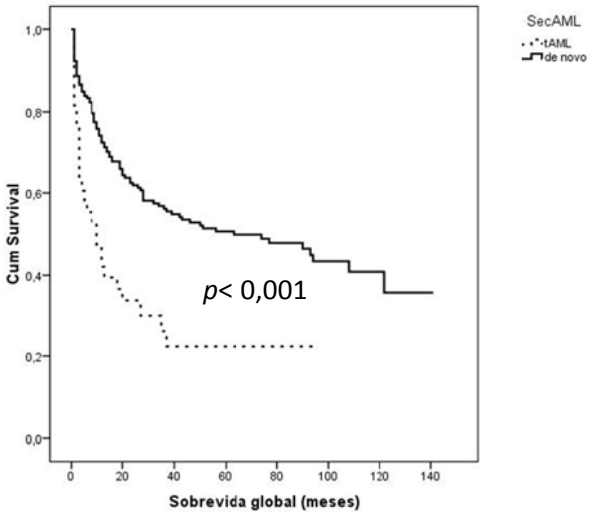


Figura 4.3: Sobrevida global dos doentes com LMA de novo e secundária à terapêutica

No grupo de LMA identificados, 38 correspondiam a casos de LMA relacionada com o tratamento o que representa cerca de 23,1% de todos os casos de LMA.

Comparando os casos de LMA relacionados com o tratamento e a LMA de novo (tabela 4.3), verificamos que objectivamente o primeiro subgrupo corresponde a doentes menos jovens com uma mediana de idade ao diagnóstico de 56 anos (p< 0,001). Estes casos encontravam-se mais frequentemente associados à presença de alterações citogenéticas (p= 0,009). Estratificando os doentes por grupo de risco

utilizando a escala SWOG, este grupo de doentes incluía mais doentes de prognóstico desfavorável (p=0,042).

Tabela 4.3: Caracterização dos doentes com LMA de novo comparativamente aos pós terapêutica

	***de novo* AML**	**t-AML**	***p***
n (%)	193 (83,5)	38 (16,5)	
Género, n (%)			
masculino	86	11	0,08*
feminino	107	27	
Idade (anos)	50 (15 – 78)	56 (17 – 78)	< 0,001^{+}
Citogenética			
Anormal	74 (46,6)	27 (94,7)	0,009*
Normal	77 (39,9)	9 (23,7)	
Nao efetuada	26 (13,5)	2 (5,3)	
CBF e t(15,17)	53 (27,5)	17 (44,7)	< 0,001*
Alterações 5,7 e 8	13 (6,7)	3 (7,9)	0,912*
t(9,11)	8 (4,1)	2 (5,3)	0,845*
Complexo	16 (8,3)	5 (13,2)	0,441*
Risco citogenético (SWOG), n (%)			
Favorável	56 (30,3)	9 (23,7)	0,656*
Intermedio	97 (52,4)	14 (36,8)	0,228*
Desfavorável	32 (17,3)	11 (28,9)	0,042*
Tratamento efectuado, n (%)			
7+3	130 (67,4)	7(21,2)	<0,001*
SWOG 9126	26 (13,5)	12 (36,4)	0,001*
LPA	37 (19,2)	6 (18,2)	0,893*
Outros	0	8 (24,2)	-
Resposta Completa, n (%)	150 (84,3%)	26 (68,4%)	0,872*

*Chi-square test; + t test

Neste grupo de doentes o tratamento efectuado também divergia do tratamento padrão nas LMA. A maioria dos doentes no subgrupo das LMA secundárias efetuou tratamento com quimioterapia associada a ciclosporina (p= 0,001).

A sobrevida global foi de 10 meses. O tempo mediano até progressão foi de 6 meses.

Tabela 4.4: Análise multivariada da sobrevida global

	p	**Hazard ratio**
Idade	0,386	0,372 (95% CI 0,400 – 3,476)
Género	0,713	0,730 (95% CI 0,118 – 4,319)
Tipo tratamento inicial	0,983	0,987 (95% CI 0,280 – 3,482)
Tempo de latência	0,011	0,220 (95% CI 0,001 – 0,410)
Risco citogenético	0,031	0,260 (95% CI 0,077 – 0,882)
Tipo de quimioterapia	0,010	2,449 (95% CI 1,238 – 4,846)
Recidiva	0,440	0,384 (95% CI 0,034 – 4,344)

A análise multivariada de regressão de Cox (tabela 4.4) mostrou relação estatisticamente significativa entre a sobrevida global e o tempo que dista as duas neoplasias (tempo de latência) sendo mais favorável quanto menor o período de latência (*p*= 0,011 RR 0,220 95% CI 0,001 – 0,410). Esta relação de proteção existe também no risco citogenético em que os indivíduos com risco citogenético favorável têm melhor sobrevida (p= 0,031 RR 0,260 95% CI 0,077 – 0,882)

No entanto a adição de ciclosporina à antraciclina e citarabina não altera o mau prognóstico reservado a estes doentes (*p*=0,053) (figura 4.4).

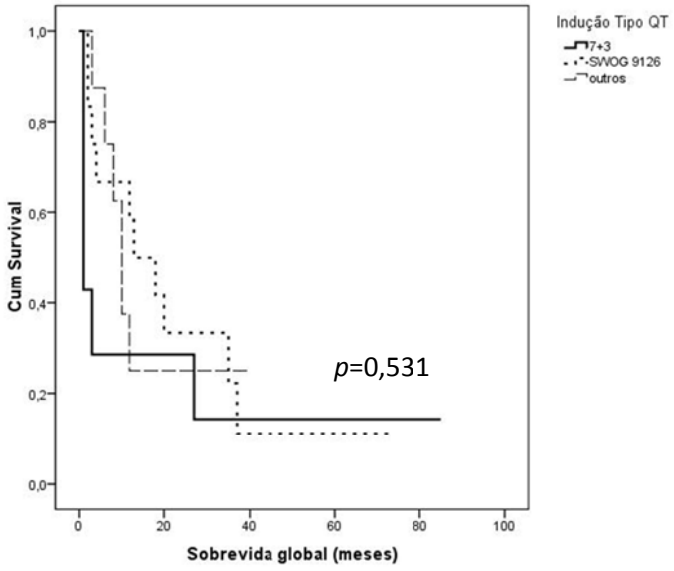


Figura 4.4: Sobrevida global dos doentes tratados com protocolo padrão comparativamente ao esquema contendo ciclosporina

LEUCEMIA LINFÓIDE AGUDA

Na série de doentes estudados 76 foram diagnosticados com Leucemia Linfóide aguda. Predominava o género masculino (n= 45; 59,2%), com mediana de idades de 37 anos (entre 14 e 79 anos).

Utilizando a classificação da OMS, a maioria dos doentes apresentava uma LLA fenótipo B, *nos* (n= 27; 37,5%). Dezoito doentes (25%) foram diagnosticados com LLA associada à t(9,22). Igual número de doentes teve diagnóstico de LLA fenótipo T. Os restantes 9 doentes apresentavam LLA B associada a outras alterações citogenéticas.

Foram definidas como alterações de mau prognóstico nas LLA do adulto a hipodiploidia, a presença de rearranjos envolvendo o gene MLL ou t(v; 11q23), a presença da t(9,22) ou do gene de fusão BCR-ABL e o cariótipo complexo (definido com

a existência de 5 ou mais alterações). Nesta série de doentes foram identificados 29 casos (38,7%) de doentes de alto risco.

O tratamento efectuado pela maioria dos doentes foi o HyperCVAD (n=57; 78,1%). Os restantes doentes fizeram outros esquemas de quimioterapia escolhidos quer pela idade (adultos jovens ou idosos) quer pela presença de fenótipo T: BFM 90 (n=7; 9,6%), vincristina e dexametasona (n=4; 5,5%), Linker (n=3; 4,1%) e BF 12 (n= 2; 2,7%). A estes esquemas de quimioterapia foi associado inibidor de tirosina cinase (imatinib) sempre que se encontrava presente o gene de fusão BCR-ABL.

Foi documentada resposta completa após indução em 53 doentes (82,8%). Uma minoria foi primariamente refratária ao esquema de indução (n= 11; 17,2%). No grupo dos respondedores, 33 doentes (62,3%) recaíram.

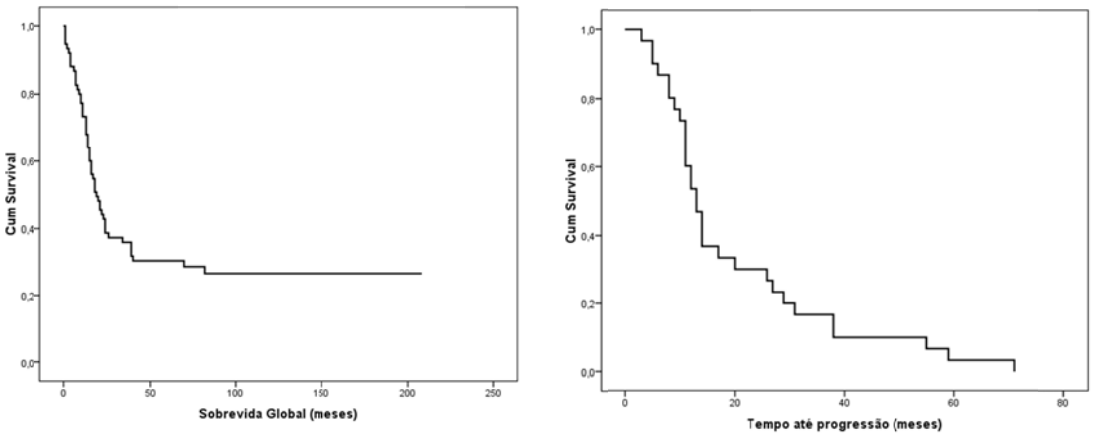


Figura 4.5 : Sobrevida global e tempo até progressão dos doentes com LLA

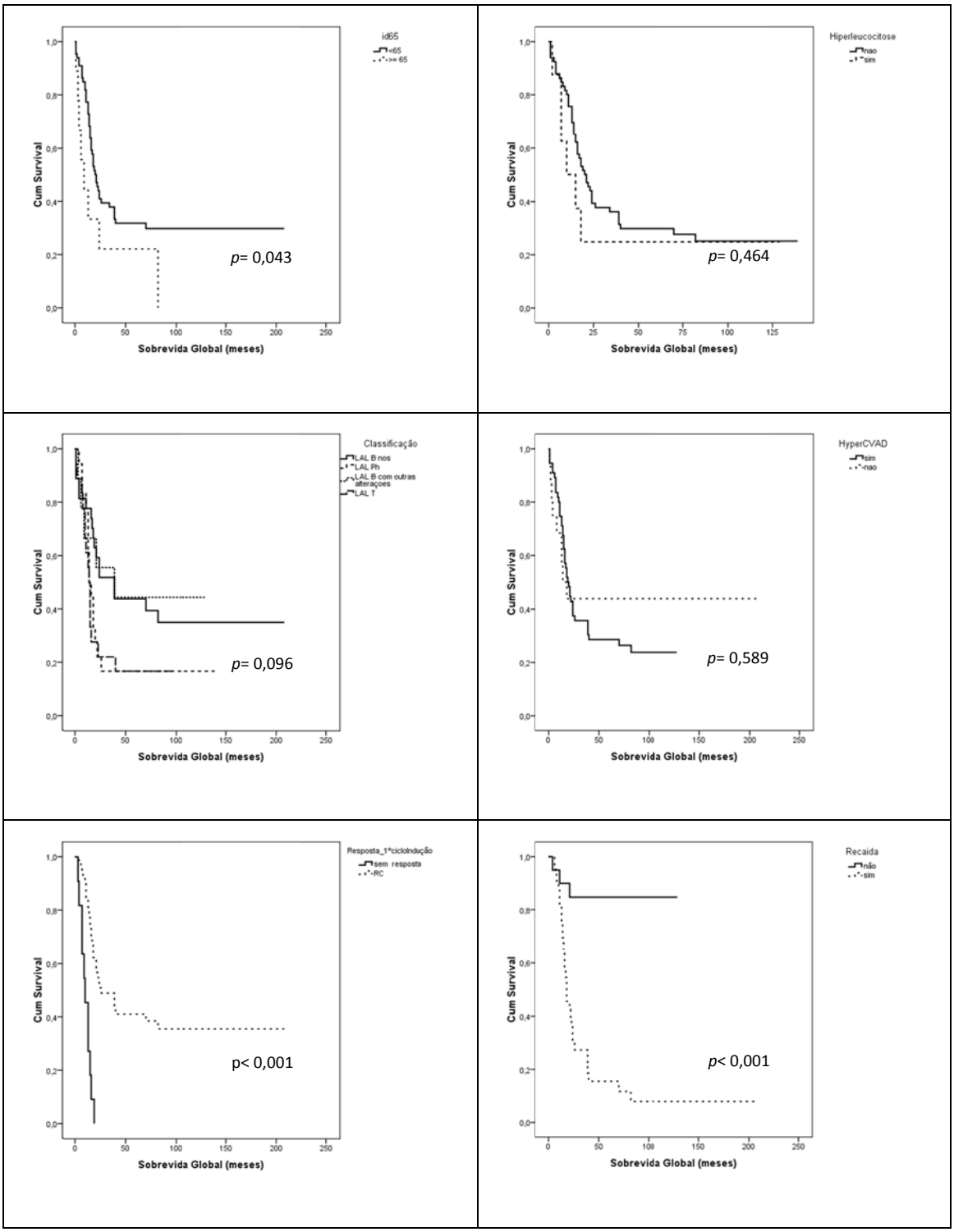


Figura 4.6: Influência da idade, hiperleucocitose, Classificação OMS, tratamento com HyperCVAD, resposta à indução e recaída na sobrevida global

O alotransplante como estratégia consolidadora de resposta foi utilizado em 21 doentes (27,6%).

A sobrevida global dos doentes foi de 19 meses. O tempo médio até progressão foi de 13 meses (figura 4.5).

Analisando alguns factores preditivos de resposta conhecidos na nossa amostra verificamos que apenas a idade, a resposta à quimioterapia de indução e a recaída da doença de base interferem de uma forma estatisticamente significativa com a sobrevida global. A hiperleucocitose, o alto risco citogenético, a presença do cromossoma Filadelfia, o tratamento efectuado e a realização de Alotransplante de medula óssea não alteram a sobrevida global (figura 4.6).

Efetuando uma análise multivariada de regressão de Cox (tabela 4.5) observamos um efeito de risco sobre a sobrevida global estatisticamente significativo para a idade superior a 65 anos (*p*= 0,042; 2,966 95% CI 1,038 – 8,475) e a presença de cromossoma Filadélfia (*p*= 0,050; 2,102 95% CI 1,000 – 4,419). A resposta à terapêutica de indução também se mostrou fator independente de sobrevida, diminuindo o risco de morte (*p*< 0,001; 0,180 95% CI 0,532 – 2,179).

Tabela 4.5: Análise multivariada da sobrevida global

	p	Hazard ratio
Idade	0,042	2,966 (95% CI 1,038 – 8,475)
Género	0,082	0,516 (95% CI 0,245 – 1,086)
Hiperleucocitose	0,928	0,947 (95% CI 0,288 – 3,112)
Cromossoma Filadelfia	0,050	2,102 (95% CI 1,000 – 4,419)
Classificação OMS	0,077	1,326 (95% CI 0,969 – 1,814)
Terapêutica com HyperCVAD	0,717	0,835 (95% CI 0,316 – 2,206)
Resposta	<0,001	0,180 (95% CI 0,532 – 2,179)
AloTMO	0,837	1,077 (95% CI 0,532 – 2,179)

POLIMORFISMOS DO GENE MDR1 E LEUCEMIAS AGUDAS

Do universo total de doentes identificados, 37 foram genotipicamente estudados para avaliação dos polimorfismos C1236T e C3435T do gene MDR1. Como grupo controlo foram usadas amostras de 160 dadores de sangue saudáveis.

Estes doentes eram na maioria do género masculino (n= 27; 73%); a mediana de idade ao diagnóstico era de 52 anos (de 18 a 72 anos).

Tabela 4.6: Frequência alélica / genotipica e equilíbrio de Hardy-Weinberg para ambos os polimorfismos estudados em controlos e casos de LA

		LA		Controlos		EHW *p*	
		n	%	n	%	LA	Controlo
C3435T	**Alelo**						
	C	39	65	185	57,8	0,178	0,413
	T	21	35	135	42,2		
	Genótipo						
	CC	11	36,7	56	35		
	CT	17	56,7	73	45,6		
	TT	2	6,7	31	19,4		
	C carriers	28	93,3	129	80,6		
C1236T	**Alelo**						
	C	42	75	190	59,4	0,449	0,395
	T	14	25	130	40,6		
	Genótipo						
	CC	15	53,6	59	36,9		
	CT	12	42,9	72	45		
	TT	1	3,6	29	18,1		
	C carriers	27	96,4	131	81,9		

A distribuição dos polimorfismos estudados entre doentes e controlos era sobreponível. As regras do equilíbrio de Hardy-Weinberg estavam presentes (tabela 4.6).

Em relação ao polimorfismo C3435T não foram encontradas diferenças estatisticamente relevantes entre os doentes e os controlos.

Tabela 4.7: Frequência alélica e genotipica dos polimorfismos do gene MDR1 e risco de LA

Polimorfismo		**Controlos (n)**	**LA (n)**	**OR (95% CI)**	***P***
C3435T	**Alelo**				
	C	185	39	*Referência*	
	T	135	21	1,36 (0,74 – 2,51)	0,300
	Genótipo				
	TT	31	2	*Referência*	
	CT	73	17	0,33 (0,05 – 1,73)	0,148
	CC	56	11	0,28 (0,04 – 1,379	0,811
	C carriers	129	28	3,36 (0,72 – 21,6)	0,092
C1236T	**Alelo**				
	C	190	42	*Referência*	
	T	130	14	0,49 (0,24 – 0,97)	**0,026***
	Genótipo				
	TT	29	1	*Referência*	
	CT	72	12	4,83 (0,60 – 103,96)	0,105
	CC	59	15	7,37 (0,94 – 156,8)	**0,030***
	C carriers	131	27	5,98 (0,81 – 122,85)	0,523

Já no polimorfismo C1236T foram encontradas diferenças estatisticamente significativas neste grupo de doentes. Assim a presença do alelo C neste polimorfismo associa-se a um maior risco de doença (HR 0,49 95% CI 0,24 – 0,97; *p*= 0,026), também estatisticamente relevante no genótipo CC (HR 7,37 95% CI 0,94 – 156,8; *p*= 0,03) (tabela 4.7)

Posteriormente e utilizando dados previamente publicados, avaliamos o efeito destes 2 polimorfismos em risco e sobrevida para Leucemia aguda numa série de populações europeias, asiáticas e da américa latina (tabela 4.8).

Tabela 4.8: Diferente incidência, prevalência e mortalidade em casos de Leucemia aguda em diferentes populações (incidência, prevalência e mortalidade expressas em ASR – "age specific rates"/ 100 000 habitantes; frequências genotipicas expressas em %)

População	Leucemia aguda			Genótipo CC	
	Incidência	Prevalência	Mortalidade	C1236T	C3435T
Chinesa	4,30	1,50	3,60	11,00	30,00
Checa	5,90	1,50	3,40	32,00	21,00
Equatoriana	6,5	8,40	5,00	-	24,90
Alemã	7,80	1,80	3,30	35,00	21,00
Húngara	6,80	1,90	4,00	33,20	22,30
Indianos	2,80	1,30	2,30	15,60	24,70
Iraniana	5,8	6,7	4,6	-	17,60
Japonesa	4,30	0,70	2,70	11,00	36,00
Polaca	5,6	17,6	3,8	-	22,00
Portuguesa	6,50	2,10	3,70	22,80	22,80
Russa	6,30	2,20	3,40	24,00	21,00
Servia	7,10	2,00	4,60	23,00	19,00
Espanhola	6,50	1,90	3,00	35,70	24,00
Turca	5,80	1,20	4,60	20,00	20,00
Reino Unido (caucasiana)	7,50	2,00	3,20	32,60	24,00

Observamos uma grande variabilidade de incidência e da prevalência de doença (ASR/100000 habitantes). A incidência variava entre 2,8 observado na India e

7,8 presente na Alemanha. A Polónia regista a maior prevalência (17,8 ASR/100000) e a menor prevalência é observada no Japão (0,7 ASR/100000).

A frequência genotípica dos polimorfismos em questão é também muito variável nas populações apresentadas.

O genótipo CC do polimorfismo C1236T apresenta um mínimo no Japão e China (11%) e um máximo em Espanha (35,7%). No polimorfismo C3435T também se observa grande variabilidade do genótipo CC estendendo-se desde o mínimo 17,6% presente no Irão e o máximo de 30% observado na China.

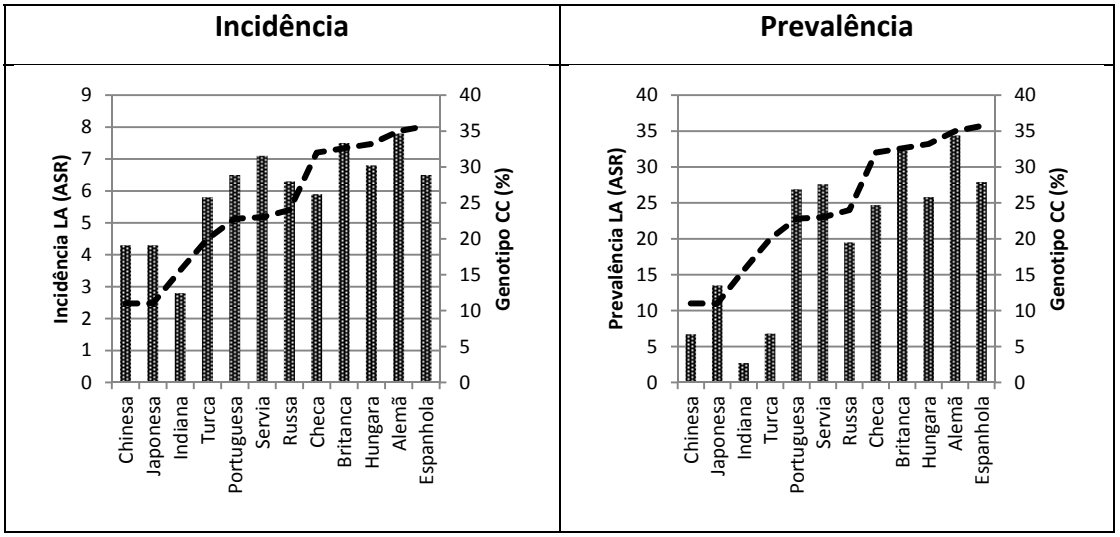


Figura 4.7: Correlação gráfica do polimorfismo C1236T com a incidência e prevalência de LA (Coeficientes de correlação: A-0,617 B-0,679)

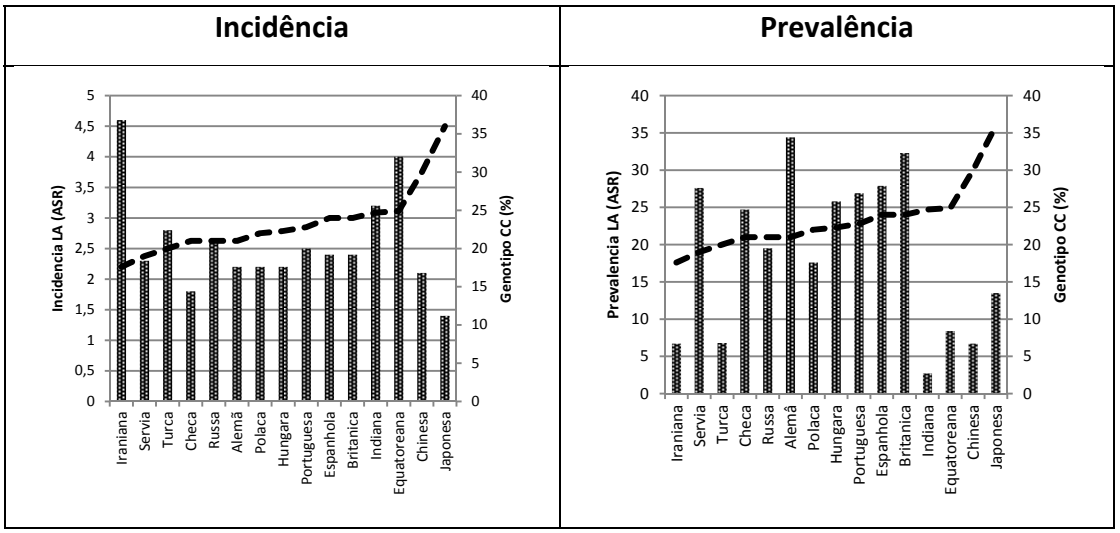


Figura 4.8: Correlação gráfica do polimorfismo C3435T com a incidência e prevalência de LA (Coeficientes de correlação: A-0,262 B-0,057)

Quando se correlaciona estes dados de incidência e prevalência de patologia com frequência genotípica, conseguimos mostrar relação de risco para Leucemia Aguda e genótipo CC no polimorfismo C1236T (figura 4.7).

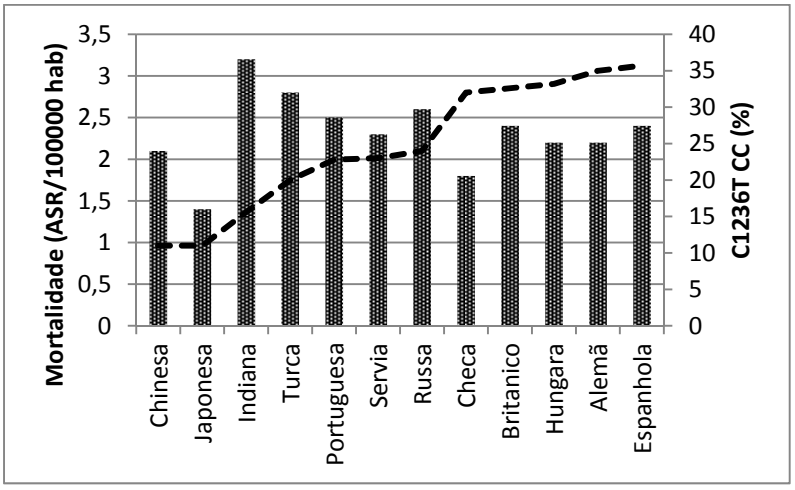


Figura 4.9: Correlação gráfica entre o genótipo 1236CC e a mortalidade em LA (Pearson -0,614; p= 0,034)

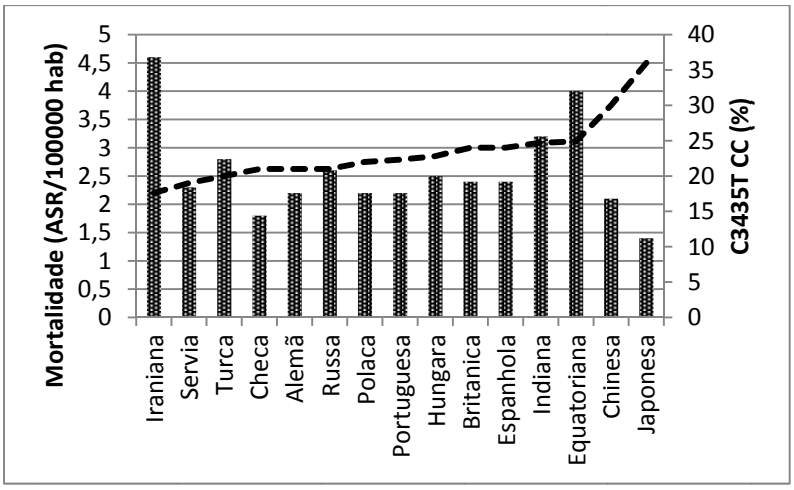


Figura 4.10: Correlação gráfica entre o genótipo 3435CC e a mortalidade em LA

Fazendo a mesma análise mas agora para o polimorfismo C3435T não foi encontrada qualquer relação (figura 4.8).

Em relação ao risco de morte foi encontrada correlação inversa (Pearson -0,614; *p*= 0,034) com o genótipo CC do polimorfismo C1236T (figura 4.9 e 4.10).

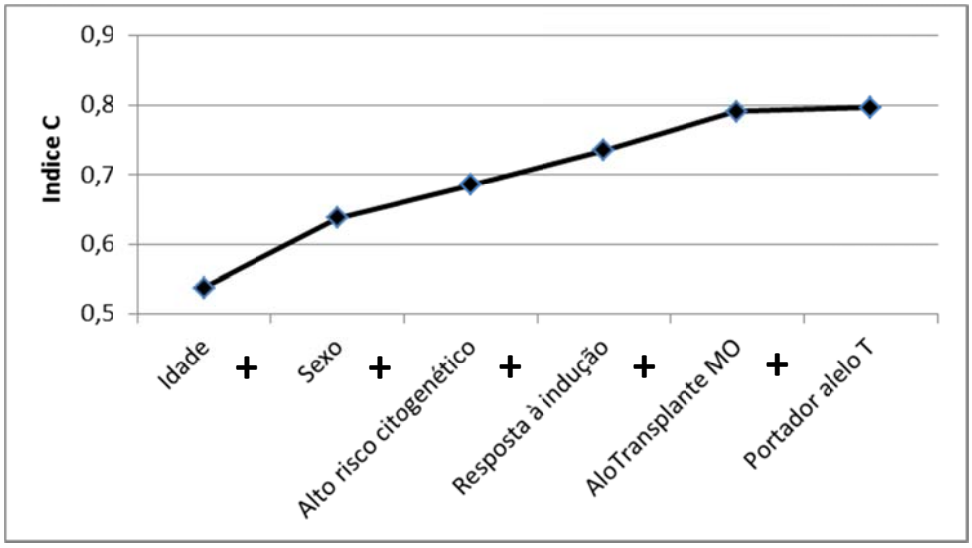


Figura 4.11: Capacidade preditiva de cada variável adicionada a anterior

Para determinar o melhor modelo preditivo de sobrevida nos doentes com LA comparamos o efeito das variáveis clinico patológicas e genéticas usando como base a análise de regressão de Cox (figura 4.11).

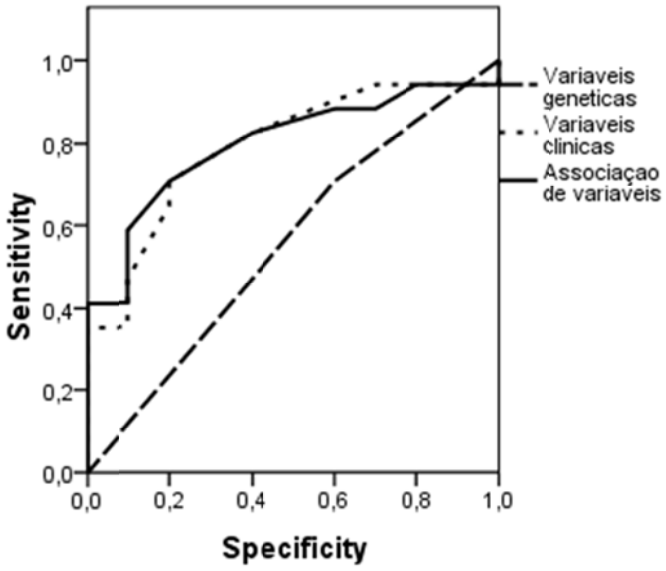


Figura 4.12: Curva ROC representando a sensibilidade e especificidade das variáveis clinica, genética e da associação de ambas

O índice C foi usado para comparar a capacidade preditiva que cada variável adicionava à variável anterior; o valor preditivo foi estabelecido usando o índice C previamente descrito por Harrell et al [233, 234], em que o valor de 1 indica a concordância perfeita.

Avaliando conjuntamente as características clinico patológicas dos doentes com LA estudados verificamos um valor de índice C de 0,791. Quando associamos a este modelo clinico as características genéticas, o valor de concordância foi aumentando, até um máximo de 0,797 com a associação em conjunto da variável portador alelo T (figura 4.12).

LINFOMA NÃO HODGKIN

No presente estudo foram englobados 138 doentes com diagnóstico de Linfoma não Hodgkin. A maioria dos doentes foi diagnosticado com LNH de alto grau, que inclui o LNH DGCB (n=62) e LNH de Burkitt (n= 3). Cinquenta e sete doentes foram diagnosticados com LNH de baixo grau, correspondendo na sua maioria a casos com LNH de centro folicular (n=43) e os restantes 14 a casos de LNH tipo MALT e LNH esplénico. Os casos de LNH de células de manto e LNH de células T periférico, nos corresponderam a uma minoria de diagnósticos. As principais características destes doentes estão resumidas na tabela 4.9.

Tabela 4.9: Principais características dos doentes com LNH

	Alto grau	Baixo grau	Células do manto	T periférico, nos
n	65	57	7	9
Género masculino	35	22	6	7
Idade (mediana)	56 (25-86)	62 (15-88)	68 (56-79)	59 (40-77)
Estadio avançado	35 (42,3%)	35 (71,4%)	6 (85,7%)	5 (62,5%)
Rituximab (1ªL)	56 (86,2%)	35 (62,5%)	7 (100%)	-----
ATMO (1ª L)	15 (23,1%)	0	2 (28,6%)	0
Resposta Completa	60 (92,3%)	45 (80,4%)	6 (85,7%)	4 (44,4%)
Recaída	14 (23,3%)	21 (41,2%)	4 (66,7%)	2 (50%)
SG (meses)	NA	222	NA	43
TTP (meses)	73	84	22	22
Óbito	12 (18,5%)	11(19,3%)	3 (42,9%)	7 (77,8%)

Todos os tipos de LNH foram mais frequentes em indivíduos do género masculino, excepto os casos de LNH de baixo grau. A idade mediana de diagnóstico situou-se no intervalo entre os 56 e os 68 anos de idade. Todos os LNH, excepto os de alto grau, foram diagnosticados em estadios avançados (estadio Ann Harbor ≥ III).

A terapêutica com rituximab em primeira linha foi utilizada na maioria dos doentes com diagnóstico de LNH de células B.

O autotransplante de medula óssea foi utilizado como estratégia de consolidação em primeira linha numa minoria de doentes com LNH de alto grau e de células do manto.

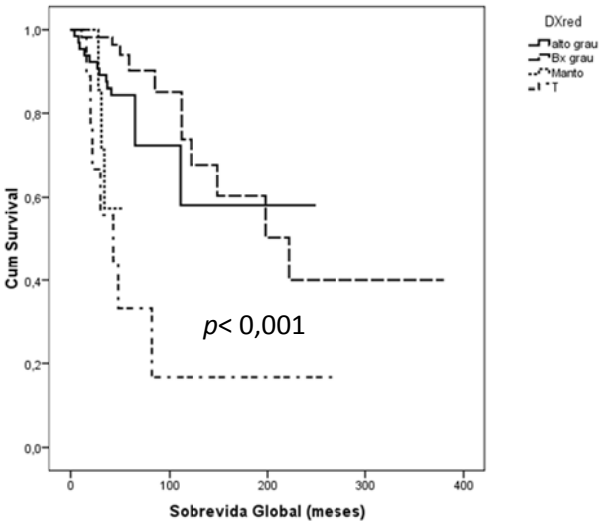


Figura 4.13: Sobrevida global dos doentes com LNH de acordo com o subtipo

A sobrevida global foi de 198 meses (figura 4.13). Analisando por subgrupos verificamos que a sobrevida global nos LNH de baixo grau foi de 222 meses e nos LNH de células T de 43 meses. A mediana de sobrevida ainda não foi alcançada nos doentes com LNH de alto grau e LNH de células do manto. Esta diferença na sobrevida é estatisticamente significativa (p< 0,001).

Analisando o TTP verificou-se que este é mais prolongado nos doentes com LNH de baixo grau (84 meses). Nos casos de LNH de alto grau é de 73 meses e nos casos de LNH de células do manto e de células T periférico é de 22 meses (figura 4.14).

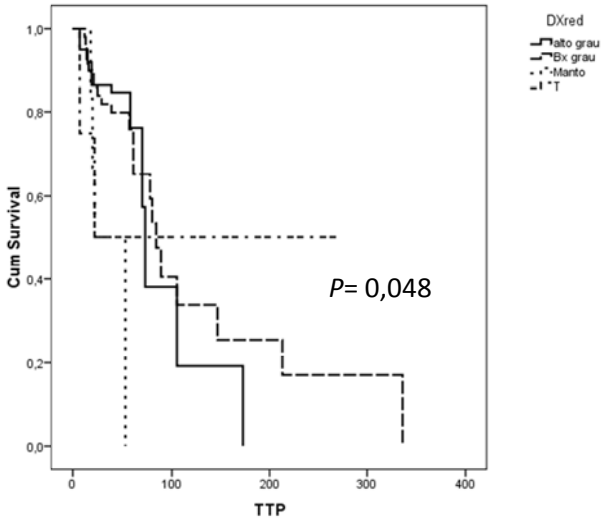


Figura 4.14: Tempo ate progressão dos doentes com LNH de acordo com o subtipo

Analisando a sobrevida global dos LNH de acordo com factores de prognóstico conhecidos (figura 4.15) encontramos influência estatisticamente significativa do género (p= 0,013), idade (p< 0,001), estadio Ann Harbor (p= 0,002) e ocorrência de recaída (p= 0,001).

O uso do rituximab em 1ª linha não alterava de uma forma estatisticamente significativa a sobrevida global (p=0,068 e p= 0,945, respectivamente) ou o tempo até progressão (p=0,963 e p= 0,241, respectivamente) quer nos linfomas de alto quer nos de baixo grau.

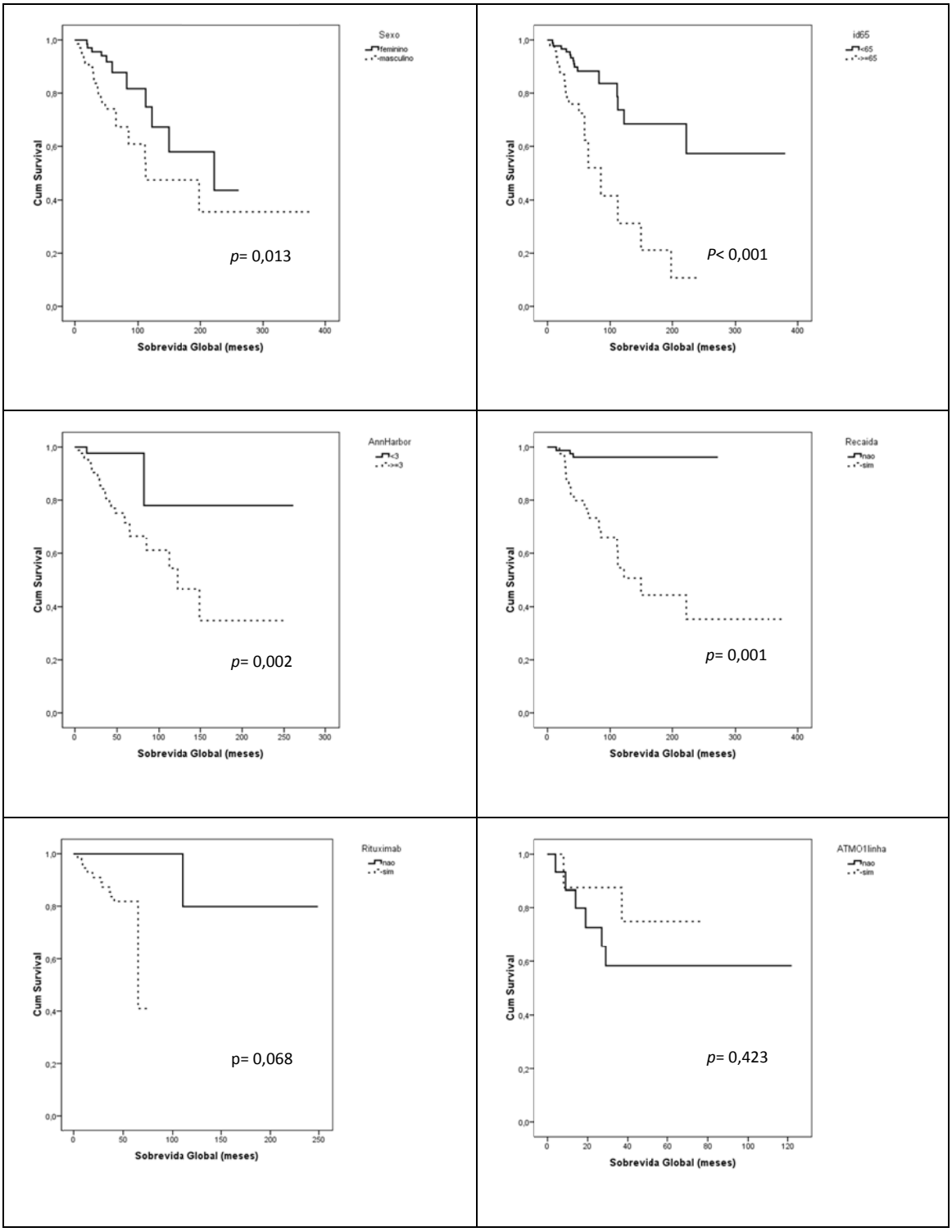


Figura 4.15: Sobrevida global de acordo com o género, a idade, o estadio Ann-Harbor, a recaída, o uso do Rituximab e a realização de ATMO

Mesmo nos doentes com estadio avançado (definido como estadio Ann-Harbor ≥3 ou IPI≥3) ao diagnóstico, a estratégia de consolidar resposta com ATMO não alterou significativamente a sobrevida.

Tabela 4.10: Análise multivariada de sobrevida

	p	Hazard ratio
Idade	0,002	3,599 (95% CI 1,607 – 8,060)
Género	0,028	2,607 (95% CI 1,110 – 6,127)
Tipo de Linfoma	0,143	1,294 (95% CI 0,916 – 1,828)
Estadio Ann-Harbor	0,072	4,012 (95% CI 0,855 – 18,191)
Rituximab em 1ª linha	0,988	1,008 (95% CI 0,349 – 2,910)
Resposta	0,018	1,920 (95% CI 1,118 – 3,297)

Efetuando uma análise multivariada de regressão de Cox (tabela 4.10) encontramos um efeito de risco sobre a sobrevida global estatisticamente significativo para a idade (*p*= 0,002; 3,599 95% CI 1,607 – 8,060), género (*p*= 0,028; 2,607 95% CI 1,110 – 6,127) e resposta ao 1º tratamento (*p*= 0,018; 1,920 95% CI 1,118 – 3,297).

POLIMORFISMOS DO GENE MDR1 E LINFOMA NÃO HODGKIN

Todos os doentes identificados com LNH foram genotipicamente estudados para avaliação dos polimorfismos C1236T e C3435T do gene MDR1. Como grupo controlo foram usadas amostras de 160 dadores de sangue saudáveis.

As principais características destes doentes já foram previamente descritas.

Tabela 4.11: Frequência alélica / genotipica e equilíbrio de Hardy-Weinberg para ambos os polimorfismos estudados em controlos e casos de LNH

		LNH		Controlos		EHW *p*	
		n	%	n	%	LNH	controlos
C3435T	**Alelo**						
	C	152	55,1	185	57,8	0,154	0,413
	T	124	44,9	135	42,2		
	Genótipo						
	CC	46	33,3	56	35		
	CT	60	43,5	73	45,6		
	TT	32	23,2	31	19,4		
	C carriers	106	76,8	129	80,6		
C1236T	**Alelo**						
	C	111	55	190	59,4	0,160	0,395
	T	91	45	130	40,6		
	Genótipo						
	CC	27	26,7	59	36,9		
	CT	57	56,4	72	45		
	TT	17	17	29	18,1		
	C carriers	84	83,2	131	81,9		

A distribuição dos polimorfismos estudados entre doentes e controlos era sobreponível. As regras do equilíbrio de Hardy-Weinberg estavam presentes (Tabela 4.11).

Tabela 4.12: Frequência alélica e genotipica dos polimorfismos do gene MDR1 e risco de LNH

Polimorfismo		**Controlos (n)**	**LNH (n)**	**OR (95% CI)**	***p***
C3435T	**Alelo**				
	C	185	152	*Referência*	
	T	135	124	0,89 (0,64 – 1,25)	0,501
	Genótipo				
	TT	31	32	*Referência*	
	CT	73	60	1,26 (0,66 – 2,39)	0,457
	CC	56	46	1,26 (0,64 – 2,48)	0,476
	C carriers	129	106	0,80 (0,44 – 1,44)	0,421
C1236T	**Alelo**				
	C	190	111	*Referência*	
	T	130	91	0,83 (0,58 – 1,21)	0,320
	Genótipo				
	TT	29	17	*Referência*	
	CT	72	57	0,74 (0,35 – 1,56)	0,394
	CC	59	27	1,28 (0,56 – 2,90)	0,519
	C carriers	131	84	1,09 (0,54 – 2,23)	0,789

Os dados obtidos neste estudo referentes à prevalência dos polimorfismos do gene MDR1 em amostras de doentes e controlos estão presentes nas tabelas 4.11 e 4.12.

Não foi encontrada influência dos polimorfismos do gene MDR1 no risco para LNH.

Com estes dados procedemos à comparação com resultados definidos para outras populações (tabela 4.13).

Tabela 4.13: Diferente incidência, prevalência e mortalidade em casos de Linfoma não Hodgkin em diferentes populações (incidência, prevalência e mortalidade expressas em ASR – "age specific rates"/ 100 000 habitantes; frequências genotipicas expressas em %)

População	Linfoma não Hodgkin			Genótipo CC	
	Incidência	Prevalência	Mortalidade	C1236T	C3435T
Chinesa	2,60	6,30	1,60	11,00	30,00
Checa	6,80	38,90	2,20	32,00	21,00
Equatoriana	6,90	17,5	3,2	-	24,90
Alemã	8,50	56,50	2,30	35,00	21,00
Húngara	5,90	27,50	2,50	33,20	22,30
Indianos	0,70	1,50	0,40	15,60	24,70
Iraniana	5,80	8,8	3,0	-	17,60
Japonesa	6,80	48,30	2,60	11,00	36,00
Polaca	5,60	2,0	2,3	-	22,00
Portuguesa	8,00	43,50	3,10	22,80	22,80
Russa	3,80	16,10	1,90	24,00	21,00
Servia	6,40	31,80	2,80	23,00	19,00
Espanhola	7,50	41,20	2,20	35,70	24,00
Turca	6,80	13,90	4,30	20,00	20,00
Reino Unido (caucasiana)	10,10	58,60	2,90	32,60	24,00

Mais uma vez se pode constatar a extrema variabilidade de incidência e prevalência (ASR/ 100000 habitantes) do LNH nas diferentes populações alvo. Também se observa uma grande variabilidade na frequência genómica dos polimorfismos estudados.

Avaliando de uma forma conjunta os dados de incidência de patologia e a frequência genómica foi possível elaborar gráficos de correlação para ambos os polimorfismos com a incidência e prevalência do LNH (figuras 4.16 e 4.17).

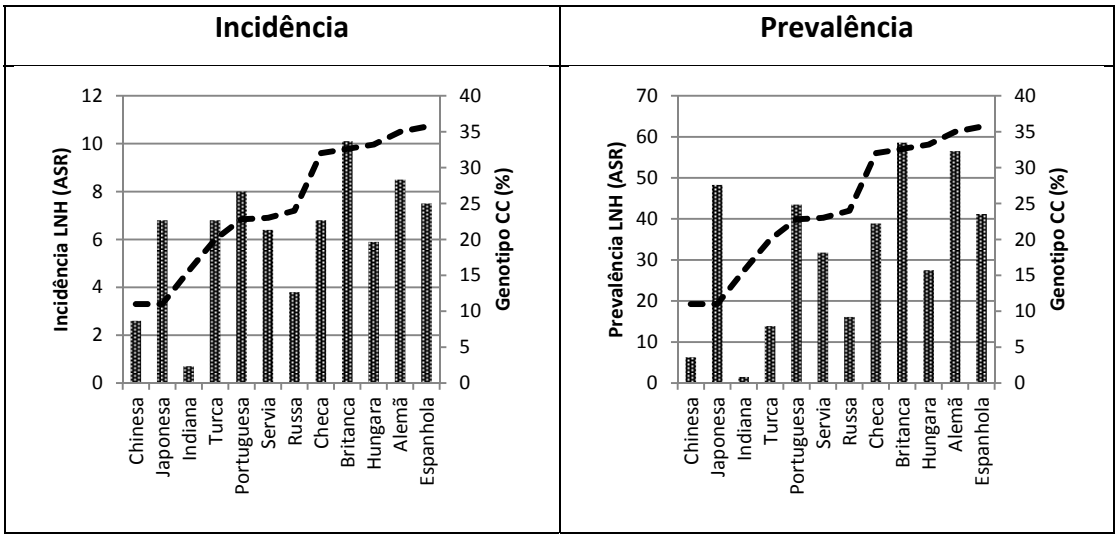


Figura 4.16: Correlação gráfica do polimorfismo C1236T com a incidência e prevalência de LNH (Coeficientes de correlação: A--0,361 B-0,307)

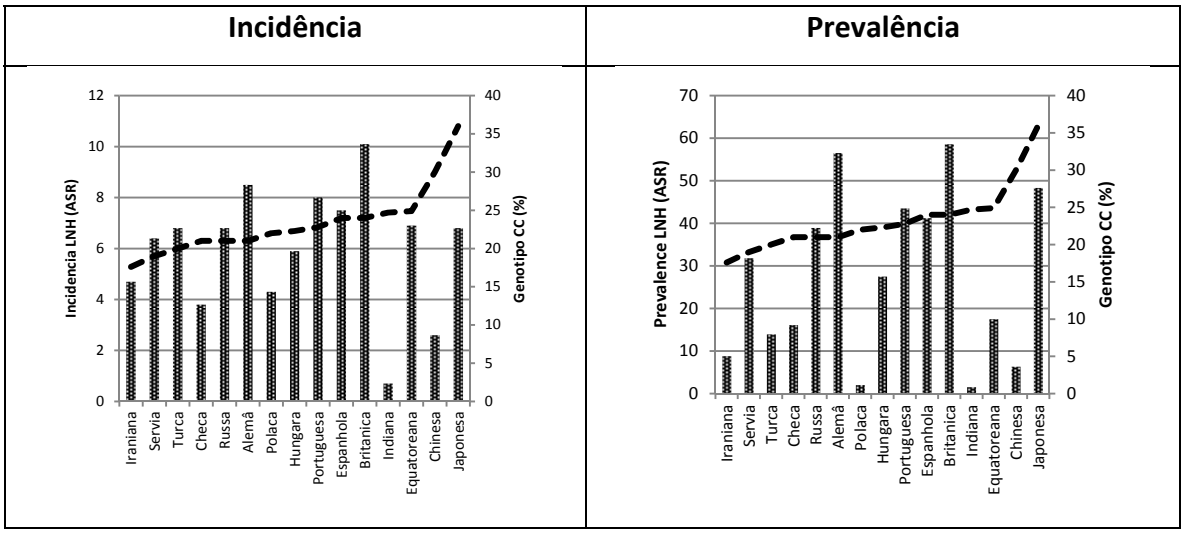


Figura 4.17: Correlação gráfica do polimorfismo C3435T com a incidência e prevalência de LNH (Coeficientes de correlação: A-0,007 B-0,024)

Estas figuras são a demonstração gráfica do índice de correlação entre o genótipo e o risco de LNH. Não foi possível definir qualquer correlação entre o risco para LNH e os polimorfismos do gene MDR1 estudados.

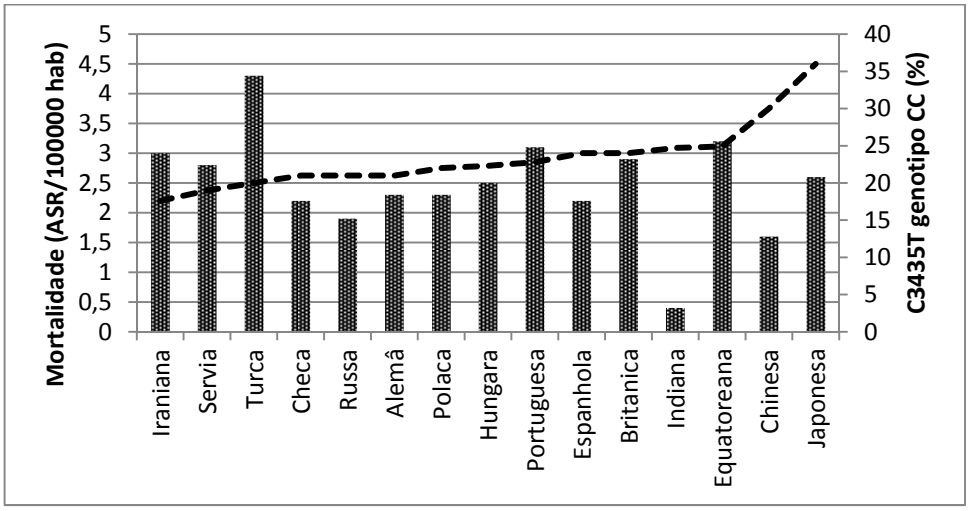


Figura 4.18: Correlação gráfica entre o genótipo 3435CC e a mortalidade em LNH (Pearson =-0,614; p= 0,034)

Em relação ao risco de morte foi encontrada correlação inversa (Pearson -0,614; *p*= 0,034) com o genótipo CC do polimorfismo C1236T. O polimorfismo C3435T (Pearson = 0,646; *p*= 0,009) correlacionava-se também com o risco de morte.

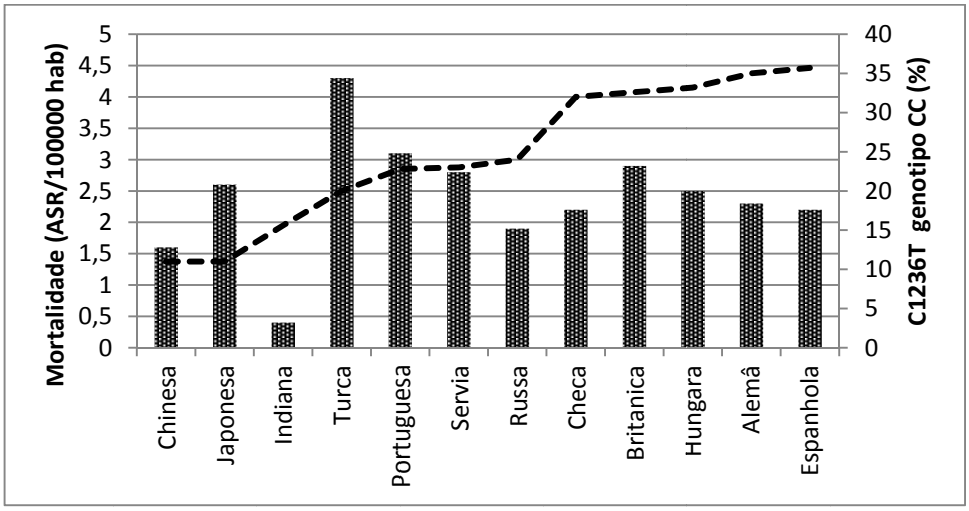


Figura 4.19: Correlação gráfica entre o genótipo 1236CC e a mortalidade em LA (Pearson = 0,646; p= 0,009)

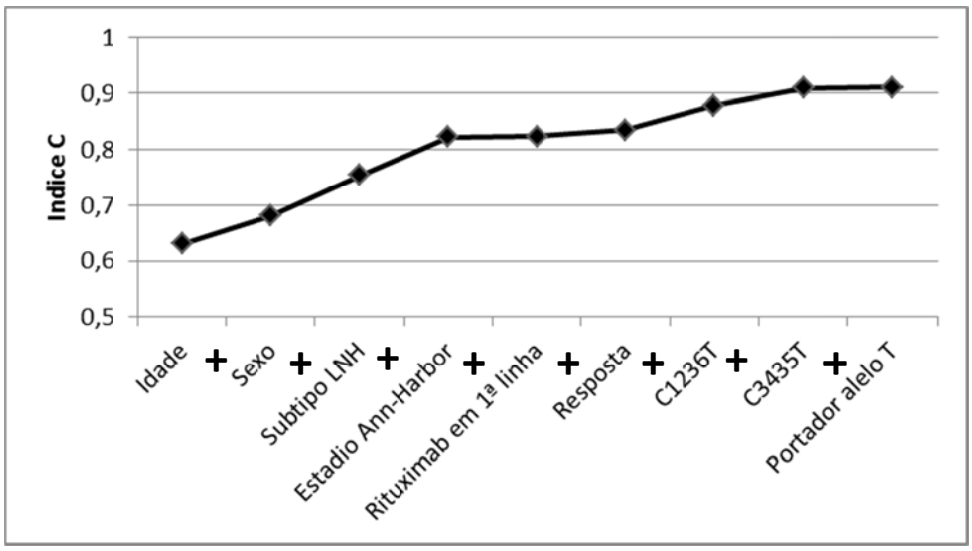


Figura 4.20: Capacidade preditiva de cada variável adicionada a anterior

Para determinar o melhor modelo preditivo de sobrevida nos doentes com LNH comparamos o efeito das variáveis clinico patológicas e genéticas usando como base a análise de regressão de Cox (figura 4.20).

O índice C foi usado para comparar a capacidade preditiva que cada variável adicionava à variável anterior; o valor preditivo foi estabelecido usando o índice C previamente descrito por Harrell et al [233, 234], em que o valor de 1 indica a concordância perfeita.

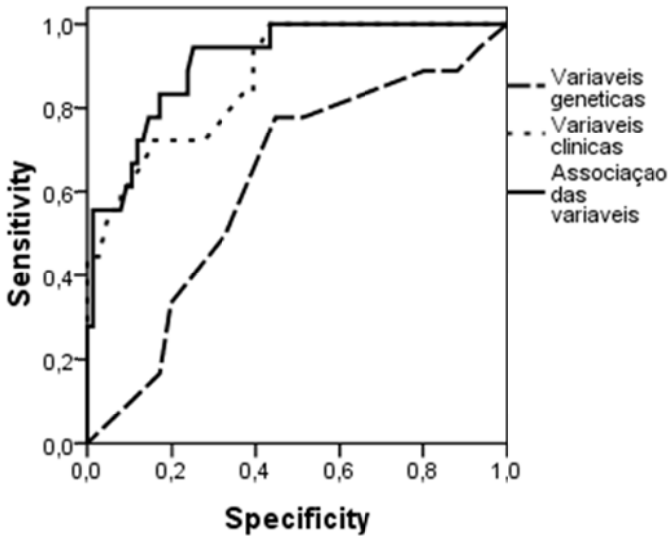


Figura 4.21 Curva ROC representando a sensibilidade e especificidade das variáveis clinica, genética e da associação de ambas

Avaliando conjuntamente as características clinico patológicas dos doentes com LNH estudados verificamos um valor de índice C de 0,835. Quando associamos a este modelo clinico as características genéticas, o valor de concordância foi aumentando, até um máximo de 0,911 com a associação em conjunto das variáveis genótipo de C1236T, genótipo de C3435T e portador alelo T (figura 4.21).

5. DISCUSSÃO

PREVALÊNCIA DE POLIMORFISMOS DO GENE MDR

Para este estudo foram avaliados um total de 326 indivíduos com patologia hematológica maligna e 160 controlos saudáveis.

A prevalência dos polimorfismos estudados do gene MDR1 – C1236T e C3435T, não divergia de uma forma estatisticamente significativa entre os doentes e saudáveis.

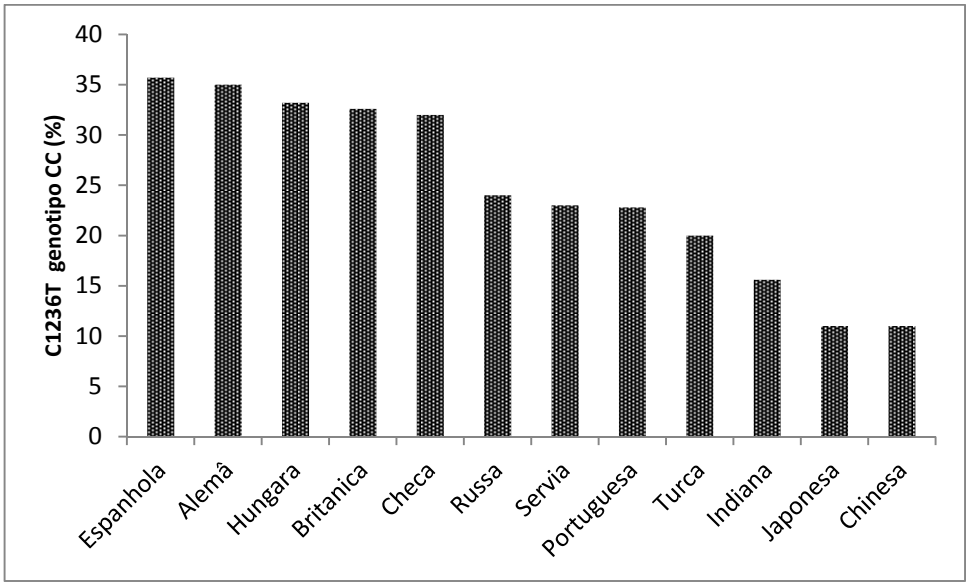


Figura 5.1: Variabilidade populacional da frequência do genótipo CC do polimorfismo C1236T

Avaliando e comparando os dados publicados previamente em relação a prevalência do genótipo CC no polimorfismo C1236T verificamos valores geralmente mais baixos nas populações da Asia e mais altos nas populações da Europa. A

prevalência vai aumentando então de este para oeste atingindo valores médios na Turquia e em Portugal (figura 5.1).

No polimorfismo C3435T valores mais altos de prevalência do genótipo CC são encontrados em populações asiáticas com descida até as populações europeias (figura 5.2).

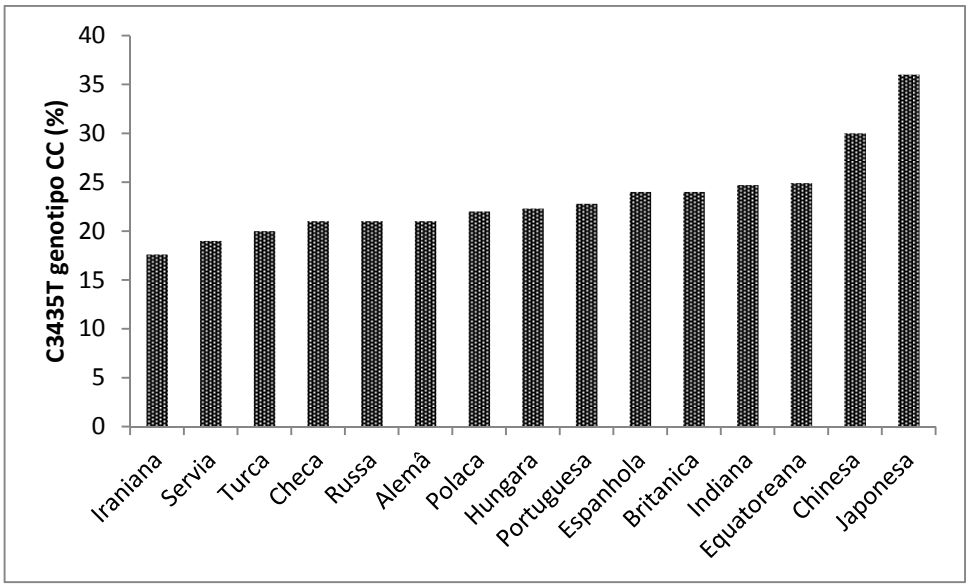


Figura 5.2: Variabilidade populacional da frequência do genótipo CC do polimorfismo C3435T

A variabilidade étnica é um dos factores mais importantes preditivo do efeito de variadíssimos fármacos. Os polimorfismos genéticos podem influenciar o metabolismo de drogas, quando envolvem por exemplo o citocromo p450, ou a distribuição e disponibilidade dos químicos como nos polimorfismos do gene MDR1 [180] .

As diferenças étnicas dos polimorfismos genéticos são fontes importantes na investigação da evolução e migração das populações [235].

Vários estudos demonstraram previamente a variabilidade étnica do polimorfismo C3435T envolvendo o gene MDR 1 [236]. Diferentes grupos de estudo se dedicaram à avaliação da frequência genotípica em diferentes populações um pouco por todos os 5 continentes [237-243]. Um destes estudos demonstrou a presença de diferenças estatisticamente significativas ($p< 0,001$) na frequência do alelo C deste polimorfismo entre a população Chinesa e Africana (envolvendo a população do Gana, Quénia e Sudão) [236] . O mesmo trabalho também demonstrou que o alelo T é relativamente raro na população africana ancestral (o genótipo TT não tinha qualquer expressão na população estudada do Gana). A expressão do alelo C não apresentava diferenças étnicas significativas [244, 245].

Estes resultados, associados ao facto do polimorfismo do C3435T estar diretamente relacionado com a expressão da P-gp [145, 237, 246, 247], sugere a avaliação deste polimorfismo como de extrema importância na farmacoterapia individualizada [248].

Relativamente ao polimorfismo C1236T há menos dados disponíveis na literatura. A variabilidade étnica deste polimorfismo está também presente em vários trabalhos desenvolvidos em diferentes populações [152, 249]. Não há no entanto dados que permitam definir o gene ancestral como para o polimorfismo C3435T.

A alteração genética condicionada pelo polimorfismo C1236T não muda nem a expressão nem altera a estabilidade do mRNA, como no caso do C3435T, originando antes um local de paragem no ribossoma o que pode alterar o enrolamento cotranslacional de um domínio da P-gp, essencial para o reconhecimento do substrato.

CORRELAÇÃO ENTRE POLIMORFISMOS E RESPOSTA À TERAPÊUTICA

Este trabalho permite concluir que os polimorfismos do gene MDR1 influenciam o risco e a sobrevida dos doentes com patologia hematológica maligna. A análise dos resultados indicados neste estudo sugerem que:

1. O genótipo 1236CC influencia o risco para leucemia aguda da população Portuguesa estudada.

2. Existe correlação entre o genótipo 1236CC e a prevalência e incidência de Leucemia aguda.

3. O genótipo 1236CC correlaciona-se com o risco de morte quer nos casos de Leucemia aguda quer em LNH.

4. O genótipo 3435CC relaciona-se com risco de morte em LNH.

Existem variados trabalhos publicados que debatem a ligação entre os polimorfismos do gene MDR1, o risco para doença e a resposta à terapêutica. Nem sempre estes resultados são coesos. A disparidade de resultados pode ser explicada pelas diferentes etnicidades, pelo tamanho da amostra e pela própria heterogeneidade dentro de uma mesma patologia. O papel dos diferentes haplótipos não pode também ser esquecido.

Em relação ao polimorfismo C1236T uma metanálise de Brissom et al, em 2015 [19] avaliou a influência destes polimorfismos no risco de Leucemia aguda linfoblástica

da criança. Foi concluído haver relação entre os polimorfismos estudados e o risco para Leucemia aguda, mas também reforçou a associação com exposição a tóxicos ambientais como tintas, pesticidas de uso domestico, insecticidas, uso de tabaco, consumo de álcool e trihalometanos. Num outro trabalho publicado em 2007 já esta relação entre os polimorfismos e o efeito leucemogénico dos pesticidas tinha sido avaliado [155].

Numa população adulta os autores Ma, L. et al apresentam uma metanálise em 2015 estudando o polimorfismo C1236T e demonstraram a sua relação com o risco aumentado para o desenvolvimento quer para Leucemia mielóide aguda quer para Leucemia mielóide cronica [250]. Estes dados vêm de encontro aos nossos resultados uma vez que na população Portuguesa se verifica um aumento do risco para leucemia aguda associado ao genótipo 1236 CC e a associação deste genótipo com a incidência e prevalência de leucemia aguda (figura 4.7).

Os polimorfismos do MDR foram associados a expressão e actividade da P-gp. Assim o polimorfismo C3435T esta directamente relacionado com a expressão da proteína, alterando a sua actividade, tendo sido demostrada uma actividade de proteína 2 vezes inferior no aparelho digestivo de indivíduos 3435TT quando comparados aos homozigotos para CC [237]. Esta diminuição da expressão leva a alterações da concentração sérica dos fármacos, substratos da P-gp, necessitando os doentes 3435TT de menor dose de terapêutica para atingir a mesma concentração sérica que os 3435CC[251, 252].

O polimorfismo C1236T não está relacionado com a expressão da P-gp mas com uma alteração na conformação proteica induzida pela alteração no enrolamento proteico, levando a uma alteração de conformação do local de ligação do substrato [253, 254].

Qualquer alteração, quer na expressão quer na funcionalidade da P-gp alterará a eficácia deste transportador. A alteração no transporte quando relacionada com a expressão da proteína poderá levar ao aumento ou diminuição do substrato no meio intracelular, aumentando ou diminuindo a sua acção sobre a célula. Se a alteração ocorrida na proteína for uma alteração conformacional que inviabilize a ligação ao substrato este irá sempre acumular-se no interior da célula.

Sabendo-se que o genótipo 3435CC do gene MDR1 está associado ao aumento da expressão da P-gp poderemos relacionar o aumento do risco de morte associado a este polimorfismo como o resultado da diminuição da eficácia dos fármacos associada a sua maior expulsão do espaço intracelular. Estes teriam menos tempo para exercer o seu efeito sobre o ciclo celular, sendo excretados do interior da célula precocemente. A diminuição no tempo de contacto com a célula originaria a sua menor acção, logo menor eficácia, logo maior risco de morte. Esta alteração seria passível de ser ultrapassada com o aumento da dose de fármaco que permitiria um somatório de acção equivalente, uma vez que estes doentes são “excretores” rápidos.

O polimorfismo C1236T encontra-se associado à alteração da capacidade de ligação ao substrato. O seu efeito associa-se ao incremento da concentração intracelular do químico. Sabemos que há vários produtos químicos potenciais leucemogénicos (pesticidas, insecticidas, tintas, derivados do petróleo, entre outros) e

sabemos também que a função principal da P-gp é proteger o organismo das agressões tóxicas externas. Se houver uma alteração na ligação do substrato este irá funcionar de uma forma deficitária, levando ao seu acumular no interior da célula, onde exercerá a sua acção tóxica.

As drogas utilizadas nos tratamentos de quimioterapia são também substratos da P-gp. Se a deficiência de estrutura do transporte dificultar a excreção dos tóxicos também vai levar ao acumular dos fármacos no espaço intracelular. Este efeito poderá ser benéfico e permitir uma maior eficácia dos fármacos com a dose padrão. Estes doentes estão no entanto sujeitos a maior toxicidade dos fármacos instituídos.

DEFINIÇÃO DO PERFIL FARMACOGENÓMICO DOS DOENTES ESTUDADOS

Com a evolução do conhecimento sobre a etiologia e a patogenia das doenças foram-se descobrindo novos factores influenciadores de resposta.

Assim sabemos que há alterações citogenéticas que condicionam a sobrevida dos doentes com Leucemia aguda. As alterações envolvendo o "core binding fator", necessário no processo da hematopoiese, são associadas a um prognóstico favorável. Neles se incluem a t(8,21) e t(16,16) / inv16. As alterações citogenéticas não balanceadas estão associadas à perda de material genético (deleções, monossomias, trissomias) e relacionam-se com um prognóstico mais desfavorável (del 7, -7, +8).

Na Leucemia Linfóide aguda as alterações citogenéticas são mais raras do que na Leucemia Mielóide aguda. A alteração mais frequentemente encontrada e associada a mau prognóstico é a t(9,22), conhecido como o cromossoma Filadelfia, que origina o gene de fusão BCR/ABL.

A idade é também associada a um pior prognóstico. Por um lado as características do individuo, mais idoso, mais frágil, com mais comorbilidades, poderão levar à necessidade de ajustar a terapêutica para doses e com fármacos menos tóxicos mas potencialmente menos eficazes. Associadamente há uma diminuição da capacidade de ultrapassar situações graves e de grande instabilidade clinica. Por outro lado a própria doença é mais agressiva, frequentemente secundária a terapêutica antineoplásica prévia e muitas vezes relacionada com resistência primária a drogas.

A resposta ao tratamento de indução é também um fator prognóstico conhecido. O seu valor encontra-se relacionado com a sensibilidade do tumor ao tratamento instituído.

A estratégia de consolidação da resposta com a realização de Alotransplante de Medula Óssea esta também associado com a doença de alto risco, influenciando desse modo a sobrevida.

Neste trabalho conseguimos definir um novo modelo preditivo de resposta nas Leucemias Agudas. O modelo englobando os critérios previamente definidos como a idade, género, risco citogenético, resposta à terapêutica de indução e realização de Alotransplante de medula óssea, tem um máximo de 0,791 no índice de concordância. Quando associamos a presença do alelo T para qualquer um dos polimorfismos o índice C sobe para 0,797.

No caso dos Linfomas não Hodgkin o principal determinante de sobrevida é o subtipo histológico. Na definição deste subtipo está incluído o fenótipo (células B, T ou NK), o índice proliferativo (indicador do "grau") e os marcadores imunocitoquímicos, para além do padrão histológico da neoplasia.

Apesar do uso genérico do estadio Ann-Harbor para todos os tipos de linfomas há depois ferramentas de estratificação mais especificas. No LNH Difuso de grandes células B, no de centro folicular e no de células do manto há índices de prognóstico específicos.

A idade apresenta-se também nos linfomas como fator de sobrevida pelas mesmas causas indicadas previamente. O uso de rituximab no tratamento dos LNH B está também associado a melhores repostas e melhor sobrevida. Como em todas estas patologias a resposta ao tratamento inicial correlaciona-se também com a sobrevida.

Avaliando o modelo clinico de resposta usando o índice de concordância obtemos um valor de 0,835. Quando a este factores se adicionam factores genéticos como o genótipo de cada um dos polimorfismos estudados e o estado de portador do alelo T o índice aumenta para 0,911.

6. CONCLUSÕES E PERSPECTIVAS FUTURAS

Neste trabalho mostra-se a importância dos polimorfismos do gene MDR1 nas doenças hematológicas malignas.

A ligação entre os polimorfismos e a patologia estende-se desde o risco até à sobrevida, influenciando a capacidade preditiva dos modelos até agora definidos.

Para as diferentes patologias estudadas fomos capazes de definir novos modelos preditivos de resposta com a associação do genótipo dos polimorfismos C3435T e C1236T do gene MDR1 aos parâmetros de prognóstico já conhecidos. Estes novos modelos ganham vantagem em especificidade e sensibilidade atingindo valores de concordância próximos da unidade.

O principal objetivo futuro prende-se com a associação destes polimorfismos a novas terapêuticas. A criação de novos fármacos mais eficazes em ultrapassar os mecanismos da P-gp ou o uso de fármacos já conhecidos com outras doses, outras associações, outros esquemas sobrepondo-se ao mecanismo da resistência primária a fármacos.

A avaliação farmacogenómica a todos os doentes previamente ao inicio do tratamento antineoplásico é também um objetivo futuro. Assim conseguiremos definir ao diagnóstico os doentes que poderão beneficiar de estratégias terapêuticas dirigidas com intenção de incrementar a eficácia terapêutica mas também de diminuir o risco de efeitos laterais.

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ANEXOS

Effect of therapy-related acute myeloid leukemia on the outcome of patients with acute myeloid leukemia

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Abstract. Therapy-related acute myeloid leukemia (t-AML) is a rare and almost always fatal late side effect of antineoplastic treatment involving chemotherapy, radiotherapy or the two combined. The present retrospective study intended to characterize t-AML patients that were diagnosed and treated in a single referral to an oncological institution in North Portugal. Over the past 10 years, 231 cases of AML were diagnosed and treated at the Portuguese Institute of Oncology of Porto, of which 38 t-AML cases were identified. Data regarding the patient demographics, primary diagnosis and treatment, age at onset of therapy-related myeloid neoplasm, latency time of the neoplasm, cytogenetic characteristics, AML therapy and outcome were collected from medical records. A previous diagnosis with solid tumors was present in 28 patients, and 10 patients possessed a history of hematological conditions, all a lymphoproliferative disorder. Breast cancer was the most frequent solid tumor identified (39.5% of all solid tumors diagnosed). The mean latency time was 3 years. In the present study, t-AML patients were older (P<0.001) and more frequently carried cytogenetic abnormalities (P=0.009) compared with *de novo* AML patients. The overall survival time was observed to be significantly poorer among individuals with t-AML (P<0.001). However, in younger patients (age, <50 years) there was no difference between the overall survival time of patients with t-AML and those with *de novo* AML (P=0.983). Additionally, patients with promyelocytic leukemia possess a good prognosis, even when AML occurs as a secondary event (P=0.98). To the best of our knowledge, the present study is the first to evaluate t-AML in Portugal and the results are consistent with the data published previously in other populations. The present study concludes that although t-AML demonstrates a poor prognosis, this is not observed among younger patients or promyelocytic leukemia patients.

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Key words: therapy-related acute myeloid leukemia, acute myeloid leukemia, cytogenetics, age

Introduction

Hematopoietic tissue malignancies are a known late side effect of previous treatments in patients that have received chemotherapy or radiotherapy for a neoplastic or other non-malignant condition (1-3). In 2008, the World Health Organization (WHO) published a review for the classification of acute leukemia (4-6) that included a novel category for neoplasms that may emerge following a previous treatment. In addition to improving treatment outcomes, the evolution of treatment options over the past decade has resulted in an increased risk of secondary malignancy (3,7). The treatment options include the use of numerous novel drugs as molecular targeted agents, novel and more intense protocols that use increased maintenance regimens and neoadjuvant therapies (8-10).

The diagnosis of therapy-related acute myeloid leukemia (t-AML) is based on the previous exposure of a patient to cytotoxic agents (4,6). In addition to this causal effect, the mechanism remains obscure; however, the mechanism appears to result from the direct mutational effect induced by prior therapy in the cells. Each cytotoxic agent may damage DNA, which may lead to various cytogenetic abnormalities and contribute to various biological characteristics in

t-AML (11-14). The time between the primary malignancy diagnosis and AML development, which is termed the latency time, varies with the treatment and cumulative dose administered, and the dose intensification of the previous cytotoxic treatment (15-18). Morphological and cytogenetic findings are also affected by previous treatments (3,7,15,19,20).

There are two distinct groups in t-AML, according to the previous treatment administered, consisting of the group of patients that were treated with alkylating agents or radiotherapy, and the group that was treated with topoisomerase II inhibitors (3,21). In the first group of patients, bone marrow myelodysplastic changes usually precede t-AML, which allows a longer latency period of 5-7 years (21,22). Poorer cytogenetic findings are also a hallmark of the group of patients treated with alkylating agents or radiotherapy, and are the cytogenetic findings are often accompanied by a complex karyotype with loss of cytogenetic material (21-24). Patients previously treated with topoisomerase II inhibitors usually present with balanced translocations. A shorter latency period, which can be ~12 months in certain cases, exhibits a rapidly progressive leukemia (21-25). The common usage of a combination of topoisomerase II inhibitors and alkylating agents led to the disappearance of these two groups (8-10).

t-AML is a rare and frequently fatal disease (2,19,23,26-28). The poor disease outcome may be explained by numerous potential factors, which include the prognosis of the primary malignancy and the toxic products of previous cytotoxic treatments that may compromise chemotherapy for AML (2,19,23,26-28). In addition to these facts, the frequent association between t-AML and poor cytogenetics appear to result in chemotherapy resistance (21,23,29).

Patients and methods

Patients. In the last 10 years, 231 patients were diagnosed with acute leukemia in the Onco-Hematology Department of the Portuguese Institute of Oncology of Porto (Porto, Norte, Portugal), the oncological referral hospital in North Portugal. Of these patients, 38 were diagnosed with t-AML. The medical records of the selected patients were reviewed in the present study. Data regarding the patient demographics, primary diagnosis and treatment, age at therapy-related myeloid neoplasm (t-MN) onset, latency time, cytogenetic characteristics, AML therapy and outcome were collected. The diagnosis of t-MN was based on the WHO 2008 classification, which concerns the previous medical history of the patient and the presence of acute leukemia laboratory findings. Bone marrow consisting of >20% myeloblasts conferred an AML diagnosis.

The ethics committee of Instituto Português de Oncologia do Porto (Porto, Portugal) approved the present study. Written informed consent was obtained from the patients for participation in the study.

Cytogenetics. G-banding was performed on bone marrow samples obtained from the patients using standard techniques (30). The cytogenetic results were presented according to the International System for Human Cytogenetic Nomenclature (31). Patients were then analyzed according to cytogenetic risk subgroups, as defined by the Southwest Oncology Group (SWOG) (32).

Statistical analyses. An analysis of the data was performed SPSS software for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA). Differences in proportions were evaluated using the χ^2 test. The probability of survival was calculated. The means and life tables were computed using the product limit estimate of Kaplan-Meier and analyzed by the Breslow (generalized Wilcoxon) test, which is a statistical test for equality of survival distributions. $P<0.05$ was considered to indicate a statistically significant difference. The hazard ratio (HR) was also assessed using multivariate Cox regression analyses of the 1-, 3- and 5-year overall survival (OS) rates. Using the HR value, the probability (P) of survival was calculated as previously described in the literature: HR = odds = P / (1 - P); P = HR / (1 + HR) (33).

The survival duration was defined as the time between diagnosis and either mortality or time of the last clinical evaluation of the patient. Only patients achieving complete remission were evaluated for disease-free survival, which was defined as the time between diagnosis and relapse. The latency interval was defined as the time period between first cytotoxic therapy and t-MN diagnosis.

Results

Patients. In total, 231 adult patients with AML were identified. The median age at diagnosis was 51 years (range, 15-78) and 58% (n=134) were females. t-AML was identified in 38 patients and the remaining 193 were diagnosed with *de novo* AML. Patients diagnosed with *de novo* AML were younger compared with t-AML patients (median age, 50 vs. 56 years; P<0.000). *De novo* AML and t-AML were more prevalent in females than in males (55.4 vs. 71.1%), but this was not a statistically significant difference (Table I).

Cytogenetics. An abnormal karyotype is the most frequent finding in t-AML patients when compared with AML patients (94.7 vs. 46.6%; P<0.000; Table I). Although there were no significant differences between the prevalence of cytogenetic abnormalities when stratifying patients using the SWOG score, t-AML patients were more frequently placed in the unfavorable risk score group than AML patients (28.9 vs. 17.3%; P=0.042). Between the favorable and intermediate risk groups, no differences were found (Table I).

When analyzing the cytogenetic alterations in t-AML by comparing the solid tumors and the hematological malignancy groups, there were no significant differences. The loss of cytogenetic material was more frequent among t-AML patients following hematological malignancies. Balanced translocations, including core binding factor (CBF) abnormalities, were more frequent subsequent to diagnosis with solid tumors (Table II).

Previous disease and treatment. The majority of patients with t-AML were previously diagnosed with a solid tumor (n=28; 74%). The most common solid tumor was breast cancer (n=15; 39.5%), followed by uterine cancer (n=4; 10.5%) and prostate cancer (n=2; 5.3%). In total, 7 cases (18.4%) were associated with several neoplastic conditions, each unique to a single

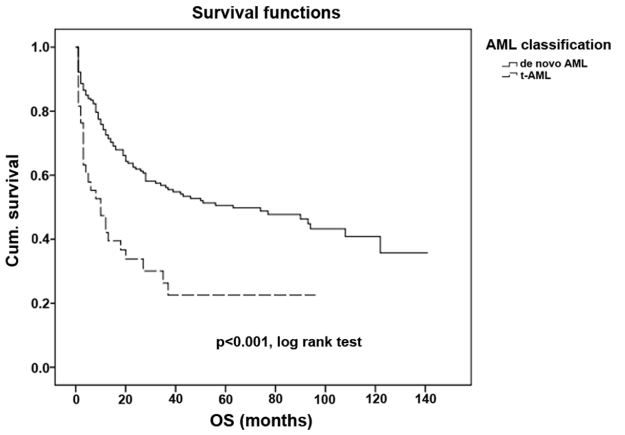

Figure 1. Overall survival time in *de novo* AML and in t-AML. t-AML, therapy-related acute myeloid leukemia; Cum., cumulative; OS, overall survival.

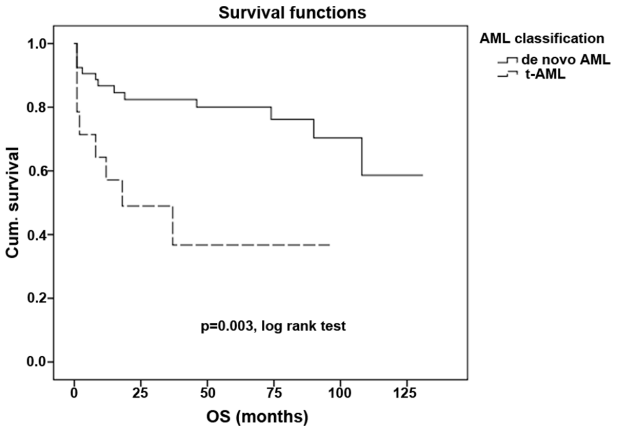

Figure 2. Overall survival time in *de novo* AML and in t-AML with core binding factor abnormalities. t-AML, therapy-related acute myeloid leukemia; Cum., cumulative; OS, overall survival.

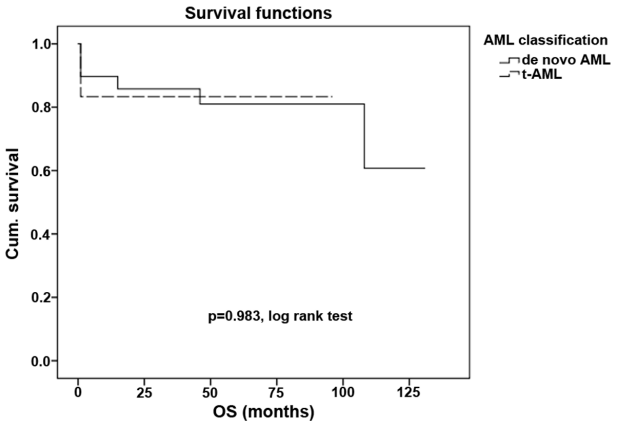

Figure 3. Overall survival time in *de novo* AML and in promyelocytic t-AML. t-AML, therapy-related acute myeloid leukemia; Cum., cumulative; OS, overall survival.

case. In the hematological malignancies group, a previous lymphoproliferative disorder was identified in all patients, with non-Hodgkin's lymphoma observed in 8 patients (21.1%) and Hodgkin's lymphoma in 2 cases (5.3%).

Chemotherapy alone was performed on 15 patients (39.5%), 13 patients (34.2%) were treated only with radiotherapy and the remaining patients had received previous chemo- and radiotherapy. The majority of patients treated with chemotherapy alone were treated with a combination of alkylating agents and topoisomerase II inhibitors (n=12; 80%). Only 3 patients (20%) were treated with alkylating agents. The median time between the first antineoplastic treatment and t-AML was 3 years (range, 1-19 years).

According to the previous treatment received, a shorter latency time was observed in the group treated with the combination of chemo- and radiotherapy, which had a median latency time of 3.5 years (range, 2-13). However, the differences between the patients treated with chemotherapy alone, radiotherapy alone or a combination of the two lacked significance (P=0.619). The complete response to induction treatment was similar in *de novo* and therapy related AML groups (P=0.872).

Survival. The median follow-up time of all patients was 23 months (range, 1-141 months). The median OS time in the entire cohort (n=231) was 39 months [95% confidence interval (CI), 10.458-67.542] and the OS rate in the subsequent 5 years was 45.9%.

The outcome of patients with t-AML is significantly poorer when compared with the outcome of patients with *de novo* AML (P<0.001; Fig. 1), with median OS times of 10 and 64 months, respectively. The OS rates at 5 years were 22.6 and 50.6% in patients with t-AML and *de novo* AML, respectively.

Cytogenetics and age are the strongest prognostic markers for the outcome in *de novo* AML. In the present study, cytogenetics (P=0.009; 95% CI, 1.042-1.336) and age (P=0.008; 95% CI, 1.153-2.616) were of independent prognostic value in all patients. However, in the t-AML cohort, cytogenetics (P=0.392; 95% CI, 0.862-1.459) and age (P=0.517; 95% CI, 0.539-3.423) lacked prognostic value.

Other core binding factor (CBF) abnormalities that are often associated with a good prognosis often lose this association in patients with secondary leukemia (P<0.001; Fig. 2). However, in the t(15,17) group of patients, even those with t-AML demonstrated the preservation of a good prognosis, with no statistically significant difference in survival compared with the *de novo* AML subgroup (P=0.983; Fig. 3).

An older age is associated with a poorer outcome in patients with t-AML. By analyzing the study population in two subgroups, one containing patients of ≤50 years and another containing patients of >50 years, the outcome of t-AML patients in the younger subgroup was similar in t-AML and *de novo* AML patients (P=0.98; Fig. 4). Older patients with t-AML are more likely to experience a poor prognosis when compared with their *de novo* counterpart (P=0.006; Fig. 5).

Multivariable Cox-regression analyses demonstrated a significant adverse impact on the 5-year OS rate of t-AML patients (P<0.001; HR, 3.363; 95% CI, 1.951-5.796), a significant HR of complete response following induction treatment (P<0.001; HR, 5.376; 95% CI, 3.303-8.750) and a significant occurrence of relapse (P<0.001; HR, 2.827; 95% CI, 1.790-4.466; Table III).

Table I. Characteristics of patients with *de novo* AML and t-AML.

Characteristic	*De novo* AML, n (%)	t-AML, n (%)	P-value
Total	193 (83.5)	38 (16.5)	
Gender			
Male	86	11	0.08*
Female	107	27	
Age, years (range)	50 (15-78)	56 (17-78)	<0.000+
Cytogenetics			
Abnormal	74 (46.6)	27 (94.7)	0.009*
Normal	77 (39.9)	9 (23.7)	
Missing	26 (13.5)	2 (5.3)	
CBF	53 (27.5)	17 (44.7)	<0.000*
5,7 and 8 alterations	13 (6.7)	3 (7.9)	0.912*
t(9,11)	8 (4.1)	2 (5.3)	0.845*
Complex	16 (8.3)	5 (13.2)	0.441*
SWOG risk score			
Favorable	56 (30.3)	9 (23.7)	0.656*
Intermediate	97 (52.4)	14 (36.8)	0.228*
Unfavorable	32 (17.3)	11 (28.9)	0.042*
Complete response	150 (84.3)	26 (68.4)	0.872*

*χ^2 test; +t test. AML, acute myeloid leukemia; t-AML, therapy-related acute myeloid leukemia; CBF, core-binding factor; SWOG, Southwest Oncology Group.

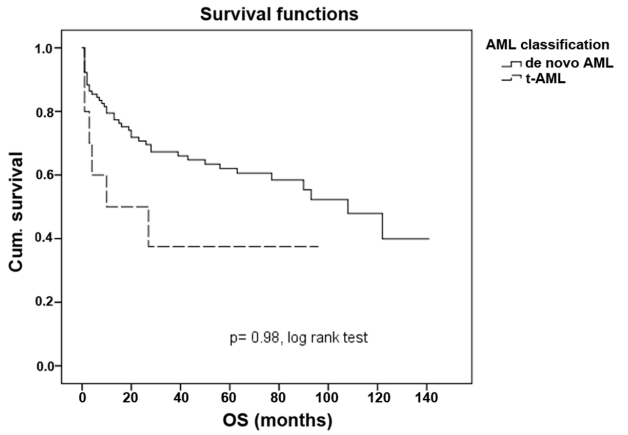

Figure 4. Overall survival time in patients younger than 50 years in *de novo* AML and in t-AML. t-AML, therapy-related acute myeloid leukemia; Cum., cumulative; OS, overall survival.

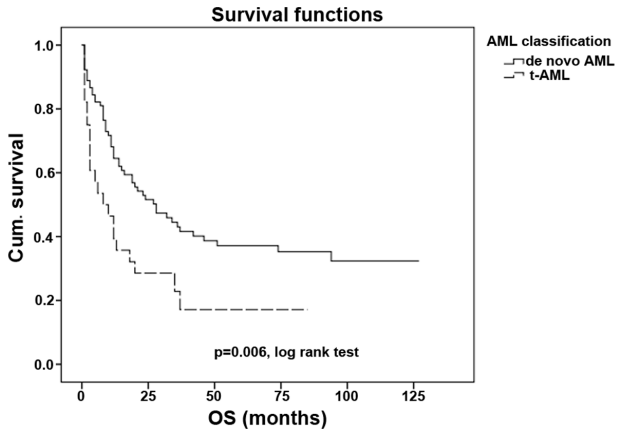

Figure 5. Overall survival time in patients older than 50 years in *de novo* AML and in t-AML t-AML, therapy-related acute myeloid leukemia; Cum., cumulative; OS, overall survival.

By independently analyzing AML and the SWOG risk score as prognostic factors, and comparing the HR for OS rate at 1, 3 and 5 years, a constant and gradual increased HR was observed for the t-AML group (Fig. 6). This trend was not observed in the same analysis for the SWOG risk, which demonstrated a decreasing HR over the years. The 5-year OS rates demonstrate a separation in the two curves, which becomes even clearer over time. The mortality risk in the t-AML group at 1 year is 75.6%, and this increased to 76 and 77% at 3 and 5 years, respectively. In the SWOG group, the mortality risk decreased between 60% at 1 year and 52% at 3 and 5 years (32).

Discussion

Long-term side effects of previous cytotoxic treatments are one of the most challenging problems faced by oncologists (2,3,7). The improved survival rates associated with the most efficient novel drugs allow patients to survive the primary cancer and develop a secondary malignancy induced by the cell toxicity of the primary treatment. Numerous studies have now been conducted to characterize this particular type of acute leukemia (2,8,19,28,34); however, to the best of our knowledge, the present study is the first

Table II. Cytogenetic abnormalities, according to the type of tumor.

Karyotype	Solid tumor, n (%)	Hematological malignancy, n (%)	P-value
Total	28 (100.0)	8 (80)	
Normal	6 (21.4)	3 (30)	0.355
CBF and balanced translocations	17 (60.7)	2 (20)	0.743
Alterations on chromosomes 5,7 and 8	2 (7.1)	1 (10)	0.629
Complex	3 (10.7)	2 (20)	0.303

P-values were calculated using the χ^2 test. CBF, core-binding factor.

Table III. Multivariate analyses of 5-year overall survival.

		5-year overall survival	
Factors	P-value	HR	95% CI
t-AML	<0.001	3.363	1.951-5.796
Age	0.065	1.523	0.974-2.382
Gender	0.736	0.928	0.601-1.433
SWOG risk score	0.864	1.048	0.614-1.787
Complete response	<0.001	5.376	3.303-8.750
Relapse	<0.001	2.827	1.790-4.466

HR indicates the risk of mortality within 5 years; HR, hazard ratio; CI, confidence interval; t-AML, therapy-related acute myeloid leukemia; SWOG, Southwest Oncology Group.

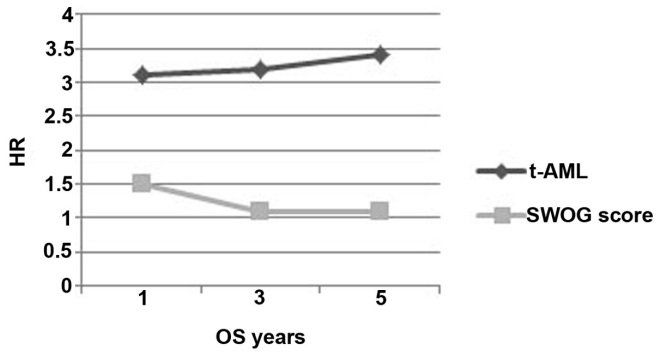

Figure 6. t-AML and SWOG score risk as independent prognostic factors: Comparing HR within the 1, 3 and 5 year timeline. t-AML, therapy-related acute myeloid leukemia; HR, hazard ratio; OS, overall survival; SWOG, Southwest Oncology Group.

to analyze therapy-induced secondary malignancy in Portuguese patients.

The frequency of t-AML in the present cohort was 16.5% (8,27), which is increased compared with previously reported data from other populations. This may be explained by the focused activity of the present hospital, an oncology referral center, which may lead to a clustering of all oncological conditions.

The response to the induction regimen was not significantly different between patients with t-AML and those with *de novo* AML. This suggests that t-AML patients do not demonstrate a worse response to chemotherapy compared with *de novo* AML patients, and also suggests that the comparably poor outcome of patients with t-AML is likely to be a result of toxicity or relapse from acute leukemia or the initial tumor (35).

Cytogenetics acts as a good prognostic marker in patients with AML (8,23,24,27,36-38). Patients with t-AML are more prone to a poor prognosis and cytogenetic findings. Core binding factor abnormalities are associated with an improved outcome in patients with AML (39-41). However, in the present cohort, t(15,17) appeared to have a good effect on the outcome of disease, even in patients with t-AML (42-45).

t-AML patients are generally older than patients with *de novo* AML. Older age has previously been linked with a poorer prognosis (42). Comorbidities and primary resistance disease have been associated with a poorer outcome (43-45). By analyzing the present data, younger t-AML patients were concluded to behave equally to *de novo* AML patients of the same age. However, older t-AML patients demonstrated a significantly poorer outcome compared with *de novo* AML patients of the same age.

In the present study, t-AML was a solid independent prognostic marker, persisting over 5 years, and was associated with a trend of increased HR. By contrast, the SWOG risk score behaved differently, losing relevance as a prognostic marker over time. A possible explanation for this observation may be that the prognostic relevance of the SWOG risk is scored only at diagnosis and not following the onset of relapse, when other cytogenetic abnormalities may occur, *de novo* or in association with the initial anomaly. From the present study,

t-AML may be concluded to be a serious late side effect of a previous oncological treatment. The incidence of t-AML may be increasing due to the use of more effective therapeutic strategies, which may lead to an increased life expectancy, therefore making it possible for late side effects to appear.

Despite the poor prognosis of t-AML, certain groups of patients may be identified that have a similar outcome to *de novo* AML patients (46-48). Promyelocytic leukemia continues to demonstrate a good prognosis, even when appearing as a secondary event following a previous malignancy (49,50); this is perhaps due to the therapy used in this particular leukemia type, including specific target agents in addition to chemotherapy.

Improvements to the outcome of t-AML depend on a deeper understanding of the disease etiology, and also depend on the use of novel drugs with increased efficacy and limited toxicity, and a preventive strategy (27). Additional studies are required, particularly to better characterize this subset of patients. Clinical trials that use novel drugs often exclude t-AML patients by omitting secondary leukemia from novel drug approval.

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Oncology Reports

SWOG pretreatment risk criteria in Acute Myeloid Leukemia – predictive or prognostic factor?

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Abstract

Acute Myeloid Leukemia is a clonal haematological malignant condition and pretreatment risk criteria implications as predictive or prognostic factors are constantly under evaluation.With this study the authors’ intent to characterise Portuguese acute myeloid leukaemia (AML) patients and to evaluate the clinical outcome associated with SWOG coding pretreatment risk criteria / cytogenetic score.In the period 2002-2010, 225 patients were diagnosed with AML at Instituto Português de Oncologia do Porto, Portugal. From this we selected 185 younger than 65 years. They were treated using common association of cytarabine and anthracycline, associated with cyclosporine when bone marrow dysplasia was observed. The median survival of 24 months was observed in this group. Patients were divided in subgroups according to SWOG pretreatment risk criteria. The authors observed a statistically significant association of non-favourable SWOG coding with female gender (p= 0,025; RR 3,632 95% CI 1,113-11,852), indication for allogeneic bone marrow transplantation (p= 0,023; RR 1,317 95% CI 1,184-1,465), complete response achievement (p= 0,013; RR 1,385 95% CI 1,232-1,556) and relapse risk (p= 0,048; RR 3,181 95% CI 0,966-10,478). Furthermore, SWOG pretreatment risk criteria also significantly influenced global OS (p= 0,003) and OS at 5 years (p= 0,001). Multivariate Cox regression analysis supported the response to induction therapy (OS 3 years: p = 0,011; 0,385 95% CI 0,184 – 0,806; OS 5 years: p = 0,012; 0,388 95% CI 0,597 – 1,994), consolidation (OS 3 years: p = 0,005; 0,328 95% CI 0,150 – 0,720; OS 5 years: p = 0,002; 0,308 95% CI 0,144 – 0,657)and the diagnosis of therapy related acute myeloid leukemia (OS 3 years: p = 0,016; 2,756 95% CI 0,486 – 1,281; OS 5 years: p = 0,031; 2,369 95% CI 1,081 – 5,189) as prognostic factors and this was not confirmed for SWOG pretreatment risk criteria.The authors conclude that the reproducibility of the application of the SWOG pretreatment risk criteria may not be available as prognostic factor in every acute leukemia population. However, its application as predictive factor of response has been confirmed in our population.

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Manuscript

SWOG pretreatment risk criteria in Acute Myeloid Leukemia – predictive or prognostic factor?

Espirito Santo A.[1,2], Chacim S. [1], Ferreira I. [1], Leite L. [1], Moreira C. [1], Pereira D. [1], Dantas M. [1], Nunes M. [1], Viterbo L. [1], Moreira I. [1], Martins A. [1], Oliveira I. [1], Domingues N. [1], Mariz J. [1], Medeiros R. [3,4,5,6,7]

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Keywords
Acute myeloid leukemia; treatment; outcome; cytogenetics; prognosis

Running title
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Abstract

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The authors conclude that the reproducibility of the application of the SWOG pretreatment risk criteria may not be available as prognostic factor in every acute leukemia population. However, its application as predictive factor of response has been confirmed in our population.

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Introduction

Acute leukemias are clonal malignant disorders arising from the primitive pluripotent hematopoietic cell, characterized by impaired proliferation of leukemic progenitors (1, 2). They are characterized by recurring chromosomal aberrations and gene mutations, with contribution of epigenetic modifications(3), crucial in differentiation, proliferation and survival pathways.

According to the latest Globocan data publication referred to 2012, in the world 351 965 persons were diagnosed with leukemia (chronic and acute as well as lymphoid and myeloid) and 265 491 died from this disease. A slight male predominance is also defined by WHO (M: F ~1,4) (http://globocan.iarc.fr/Default.aspx).

The diagnosis is based on bone marrow analysis: a smear morphology with a blasts count over 20% or the existence of recurring cytogenetic abnormalities (t(8,21), inv16, t(16,16), t(15,17)) confirms diagnosis (4).

Classification of acute leukemia, previously based only on morphological and cytochemistry findings, includes since 2002 phenotypic aspects, cytogenetic and molecular characteristics, of well-known prognostic value (4).

Conventional treatment is grounded on association of antracyclines with cytarabine over last 30 years (5). Outcome depends on multiple features, including karyotype (6-9), response to induction regimen (10), age and comorbid conditions(11). AML origin – *de novo versus* therapy related, was also associated with outcome. Because cytogenetics is considered the single most important prognostic marker, predicting remission, relapse and also OS, several cooperative group trials, like United Kingdom Medical Research Council (UK MDR), Southwest and Eastern Oncology Group (SWOG/ECOG) and Cancer and Leukemia Group B (CALGB), designed risk scores based in large cohorts of patients (6, 8, 9, 12). These scores tend to divide patients in 3 groups (favorable, intermediate and unfavorable) grounded on cytogenetic findings.

Regardless all the attempts made cure rates remains disappointing, with complete response rates at first induction of 60 to 80 % (under 60 years), but with cure rates of 30 to 40% (13).

The aim of this study is to describe the Portuguese population with acute myeloid leukaemia and to assess the impact of cytogenetics in Portuguese acute leukemia population.

Material and methods

Study design: A retrospective observational study was performed including patients under 65 years, diagnosed and treated with AML at Instituto Português de Oncologia do Porto, Portugal, since 2002 until December 2010.

Patients: from 2002 until 2010, 225 patients were diagnosed with AML in our department. On hundred and twenty eight were selected for this cohort after exclusion of patients older than 65 years old and acute promyelocytic leukemia (APL) cases.

Information regarding gender, age, date of diagnosis, classification (WHO), cytogenetic abnormalities, SWOG pretreatment risk score risk(6), treatment (1st and next lines), response, consolidation, relapse, date of relapse, bone marrow transplantation (allogeneic or autologous), date of last hospital visit or date and cause of death was reviewed from medical records of previously selected patients.

Diagnosis was based in standard criteria (4, 14, 15). All cases initially classified according to French American British (FAB) classification(14) were reviewed and reclassified according to World Health Organization (WHO)(4). A blast count higher than 20 % made AML diagnosis.

Specimens were collected for morphology, immunophenotype and genetic analyses (cytogenetic and molecular biology).

Patients were treated with the classic idarubicin and cytarabine association (16, 17) or with daunorubicin, cytarabine and cyclosporine (18, 19) if bone marrow dysplasia was present.

Response criteria (complete remission, failure or relapse) used were defined by international working groups (4, 15, 20, 21).

Ethics statement: This investigation is in accordance with the principle of the Declaration of Helsinki and was approved by the local Ethical Committee.

Cytogenetics: Standard G-banding technics were performed in patients' bone marrow samples using standard techniques; cytogenetic results were presented according to the International System for Human Cytogenetic Nomenclature (22). Patients were then analysed according to cytogenetic risk subgroups defined by the Southwest Oncology Group (SWOG)(6).

Statistical analyses: Analysis of data was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 17.0). Differences in proportions were evaluated by the 2 test. The probabilities of survival were calculated, and the means and life tables were computed using the product limit estimate of Kaplan-Meier and analysed by the Breslow (generalized Wilcoxon)

test, a statistical test for equality of survival distributions. A level of $p < 0.05$ was considered statistically significant. Hazard ratio was also accessed using a multivariate Cox regression analyses at 3 and 5 year OS.

Survival duration was defined as the time between diagnosis and either death or time of the last clinical evaluation of the patient.

As defined, prognostic factor is a measurement that is associated with clinical outcome in the absence of therapy or with the application of a standard therapy; predictive factor is a measurement that is associated with response or lack of response to a particular therapy (23, 24).

Results

Patient's cohort: Since 2002 until 2010, 225 patients were diagnosed with acute myeloid leukemia at Instituto Português de Oncologia do Porto; 128 were younger than 65 years. Both sexes were equally represented with 64 patients. The median age at diagnosis was 54 years (range 17 – 65 years).

Therapy related acute myeloid leukemia represents 23,1% of all cases.

Karyotype analyses was performed in all patients; in 2,3 % (n=3) there were insufficient metaphases for karyotype analysis. A normal karyotype was the most frequent result (n=54; 43,2%). Core binding factor anomalies were detected in 17 karyotypes (13,6%). Anomalies involving chromosomes 5, 7 and 8 were detected in 18 cases (14,4%); complex karyotype was observed in 9,6% (n=12) of the studied cases. Other less frequent alterations were detected in the remaining 24 cases (19,2%).

Using SWOG pretreatment risk criteria system the intermediate group included the majority of patients (55,5%; n= 71). Thirty seven patients (28,9%) had a unfavorable risk and 17 patients (13,6%) were included in the favorable group.

Authors found statistically significant association between cytogenetic groups and age (p= 0,031), gender (p= 0,025; RR 3,632 95% CI 1,113-11,852), allogeneic stem cell transplantation indication (p= 0,023; RR 1,317 95% CI 1,184-1,465), complete response achievement (p= 0,013; RR 1,385 95% CI 1,232-1,556) and relapse (p= 0,048; RR 3,181 95% CI 0,966-10,478) (Table 1). A significant association was also met in global OS (p= 0,003) and OS at 5 years (p= 0,001).

Treatment

Complete remission was achieved in 86,7% (n= 111) of cases. Relapsed occurred in 52 patients (46,8 % of the responders; 46,5% of total patients).
Consolidation followed induction with at least one chemotherapy course (median of 4 courses; range 1 to 5) in 96 patients (86,5%). All patients of the favorable groups followed treatment after induction; 86,4% (n= 57) and 54,1 % (n= 20) of patients from intermediate and unfavorable groups respectably received consolidation therapy.
Allogeneic stem cell bone marrow transplantation was performed in 26 patients. The majority, 61,5% from all transplants were performed in the intermediate group (n=16).

Outcome
The median survival including all patients was 24 months. The OS at 3 and 5 years was of 48,4% and 44,5%.

Different factors were found to statistical significantly influence OS at 3 and 5 years, like: complete response achievement (p< 0,001 and p< 0,001, at 3 and 5 years), SWOG pretreatment risk criteria (p= 0,001 and p= 0,001, at 3 and 5 years) , presence of therapy related acute myeloid leukemia (p< 0,001 and p< 0,001, at 3 and 5 years) and the performance of consolidation therapy (p< 0,001 and p< 0,001, at 3 and 5 years). Age (p= 0,024) and therapy performed (p= 0,01) influenced OS only at 5 years.

A multivariate Cox regression analysis (Table 2) showed that OS at 3 and 5 years was influenced by the same factors. Achievement of complete response (p = 0,011; 0,385 95% CI 0,184 – 0,806; p = 0,012; 0,388 95% CI 0,597 – 1,994), therapy related AML (p = 0,016; 2,756 95% CI 0,486 – 1,281; p = 0,031; 2,369 95% CI 1,081 – 5,189) and consolidation therapy (p = 0,005; 0,328 95% CI 0,150 – 0,720; p = 0,002; 0,308 95% CI 0,144 – 0,657). Other studies variables like sex, age (younger or older than 50 years), therapy and SWOG pretreatment risk criteria did not significantly influenced outcome.

Discussion

This study is the first one, to our knowledge, to characterize Portuguese patients diagnosed with acute myeloid leukemia and their outcome.
In our study there are more therapy related AML then in other published series (25). As our hospital is an oncology centre it centralizes a large amount of cancer patients. When AML arises as a secondary malignancy they are referred to our department.
The most frequent cytogenetic finding was a normal karyotype (26, 27). Data concerning molecular findings was not available for this study. Both NPM1 and FLT3

gene mutations are now relevant for prognosis assessment (28-32). When patients of this cohort were diagnosed this molecular analysis was not wide available.

Using SWOG pretreatment risk criteria, 3 different groups can be defined with different survivals. Male sex, complete response achievement failure, risk for relapse and indication for allogeneic bone marrow transplantation are significantly associated with a not favourable cytogenetic group. Analysing the impact of age, induction response, treatment performed, consolation therapy and t-AML, in OS at 3 and 5 years we observed that all this features influence outcome. Cytogenetic risk, defined by SWOG pretreatment risk criteria, also influences OS. However when analysing this score associated to other features also linked to outcome, like complete response achievement, cytogenetics does not statistical significantly influences OS. This can means that cytogenetic groups previously defined and related to disease prognosis (7, 12, 33, 34) may be predictive factors for response, being statistically relevant for complete response to induction therapy, but not prognostic factors, having no statistically significance in OS when associated to other entities.

Predictive factor and prognostic factor are definitions many times confused and overlapped. As defined in the literature a prognostic factor is a measurement that is associated with clinical outcome in the absence of therapy or with the application of a standard therapy that patients are likely to receive; a predictive factor is a measurement that is associated with response or lack of response to a particular therapy (23, 24). The authors conclude that SWOG pretreatment risk groups may not be consistent as prognostic markers but, instead, more reliable as predictive markers for response to standard therapy, while complete response achievement is a prognostic markers as it impacts outcome.

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Table

	Total	SWOG pretreatment risk criteria			*p**	RR* (95% CI)
		Favorable	Intermediate	Unfavorable		
n (%)	128 (100)	17 (13,3)	71 (55,5)	37 (28,9)	---	---
Age (median; years)	54	44	52	53	0,031[a]	---
Female gender, n (%)	64 (50)	13 (76,5)	34 (47,9)	17 (45,9)	0,025[a]	3,632 (95% CI 1,113-11,852)
T-AML, n (%)	24 (23,1)	2 (11,8)	11 (15,5)	11 (29,7)	0,402[a]	1,914 (95% CI 0,408-9,021)
Treatment (“7+3”), n (%)	87 (68)	15 (88,2)	49 (69)	22 (59,5)	0,063[a]	3,098 (95% CI 0,848-18,014)
Consolidation, n (%)	96 (86,5)	17 (100)	57 (86,4)	20 (54,1)	0,073[a]	1,195 (95% CI 1,092-1,308)
Allogeneic stem cell transplant, n (%)	26 (20,3)	0 (0)	16 (22,5)	10 (27)	0,023[a]	1,317 (95% CI 1,184-1,465)
Complete response, n (%)	111 (86,7)	17 (100)	66 (93)	26 (70,3)	0,013[a]	1,385 (95% CI 1,232-1,556)
Relapse, n (%)	52 (46,5)	4 (23,5)	33 (50)	13 (48,1)	0,048[a]	3,181 (95% CI 0,966-10,478)
OS (months)	24	---[c]	20	14	0,003[b]	---
OS 5 years (%)	44,5	80,9	31,3	29,4	0,001[b]	---

Table 1: Characterization of the complete cohort and of the 3 different SWOG pretreatment risk criteria groups

*Cytogenetic risk score: non favorable versus favorable

[a] Chi-square test

[b] Log rank test

[c] OS in favorable group not yet reached

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Table

	3 years Overall survival		5 years Overall survival	
	p	HR (hazard ratio)	*p*	HR (hazard ratio)
Female gender	0,517	1,226 (95% CI 0,662 – 2,271)	0,312	1,359 (95% CI 0,750 – 2,464)
Age	0,934	1,027 (95% CI 0,549 – 1,922)	0,778	1,091 (95% CI 0,597 – 1,994)
Complete response	0,011	0,385 (95% CI 0,184 – 0,806)	0,012	0,388 (95% CI 0,186 – 0,809)
Therapy	0,866	0,983 (95% CI 0,809 – 1,196)	0,504	1,064 (95% CI 0,886 – 1,278)
SWOG pretreatment risk criteria	0,338	0,778 (95% CI 0,486 – 1,281)	0,541	0,866 (95% CI 0,544 – 1,378)
t-AML	0,016	2,756 (95% CI 1,207 – 6,295)	0,031	2,369 (95% CI 1,081 – 5,189)
Consolidation therapy	0,005	0,328 (95% CI 0,150 – 0,720)	0,02	0,308 (95% CI 0,144 – 0,657)

Table 2: Multivariable analyses of 5 years OS

HR (hazard ratio) indicates the risk of dead in a 5 years' timeline
Chi square test for *p* value

EXPERT OPINION

Pharmacogenetic considerations for non-Hodgkin's lymphoma therapy

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Introduction: Chemotherapy is the current standard treatment for hematological malignancies for both curative and palliative purposes. Unfortunately, in the current treatment scenario chemotherapy resistance is an issue that is know to lead to a relapse in cancer. The multidrug resistance 1 (*MDR1*) gene is often involved in drug resistance and, so far, the best studied mechanism of resistance relates to the level of P-glycoprotein (P-gp) expression on cancer cells; however, correlation with single nucleotide polymorphism (SNP) in the *MDR1* gene has also been observed via a number of different mechanisms that interfere with function and expression of P-gp.

Areas covered: This article describes the influence of P-gp expression and SNP on the *MDR1* gene in non-Hodgkin's lymphoma (NHL) and their effect on both its risk and outcome. The authors also provide a brief summary of the more important therapeutic options, which aim to overcome this drug resistance mechanism, and discuss their known mechanisms of action.

Expert opinion: There is evidence pertaining to an association between the outcome of NHL and P-gp expression. However, the authors emphasize the need for more studies to reinforce this evidence. Furthermore, there is a definite need for the therapeutic targets, which provide tumor cellular lines of interest, to be tested in humans, in order to better evaluate their toxicity and overall effect on the outcome. The ultimate aim of this research is to develop specifically designed therapies that are tailored to the intrinsic characteristics of specific patients.

Keywords: multidrug resistance 1 gene, non-Hodgkin's lymphoma, P-glycoprotein, pharmacogenomics, polymorphisms

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1. Introduction

The use of cytotoxic drugs remains the hallmark for the treatment of hematological malignancies, for curative or palliative purposes. Chemotherapy resistance is probably one of the most important causes of cancer-related death in oncological conditions. This situation results from one of several mechanisms that arise in the individual or the tumor or that can be attibuted to the influence of a combination of factors that allow the disease to overcome therapeutics. One of the tumor mechanisms is clonal differentiation [1,2]. Cancer cells have cross-resistance to several different compounds, commonly of natural origin, that share few structural or functional similarities; all these drugs are small hydrophobic molecules that enter the cell by passive diffusion across the lipid bilayer of the cell wall. Different multidrug-resistant tumor cell lines share the same biological behavior. The overexpression of P-glycoprotein (P-gp) in the cellular membrane is the most frequent cause of multidrug resistance.

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Article highlights.

- P-gp is widely expressed at low levels in almost every tissue.
- The main function of P-gp is to translocate drugs actively (ATP efflux pump) from the cytosolic inner lipid leaflet of the plasma membrane to the outer lipid leaflet. All the possible substrates are hydrophobic and amphipathic molecules.
- *MDR1* polymorphisms are thought to be related to P-gp function probably by interfering in protein folding, changing substrate specificity and interfering in its expression.
- *MDR1* polymorphisms and P-gp expression are related to NHL outcome.
- New therapeutic strategies at aim to overcome multidrug resistance are being studied.

This box summarizes key points contained in the article.

Multidrug resistance 1 (*MDR1*) gene, located on chromosome 7, was first described in 1973 in the ovarian cells of Chinese hamster mutants [3-5]. P-gp is a 170 kDa protein and is a product of the *MDR1* gene. P-gp (**Figure 1**) is a drug efflux pump that plays a role in the development of drug resistance. It is composed of two homologous halves that probably originated by gene duplication. Each one of these similar halves has six transmembrane domains, one ATP-binding site and the 'walker A' and 'walker B' motifs; the protein also includes glycosylated sites on the exterior surface that can function as antigens for monoclonal antibodies. Phosphorylation is a crucial event for drug transportation. The large variety of possible substrates, all hydrophobic and amphipathic molecules, are capable of entering the cell by passive diffusion. The P-gp–substrate interaction takes place at the intracellular level of the P-gp or in the dual phospholipidic cellular membrane because the main function of the efflux pump to actively translocate substrate from the cytosolic intracellular side to the interstitial extracellular side. P-gp is widely expressed at low levels in almost every tissue, independently of their function. However, the epithelial cells that have an excretory function generally express P-gp in higher levels, for example, the epithelial lining cells present in the colon, small intestine, pancreatic and bile ductules, kidney's excretory system and adrenal gland [5-8]. P-gp expression is observed in endothelial cells present in blood barriers (brain, testis, mammary tissue and inner ear) [7]. It is also highly expressed in the of the endometrium and in the placenta of pregnant women [9]. In all these sites where P-gp is widely present, its protective function against toxic compounds is well defined. Single nucleotide polymorphisms (SNPs) are the most frequently inherited sequence variations in a particular gene. More than 50 SNPs of the *MDR1* gene have been described [10-12]. However, three of them are known to occur frequently (10%) in various populations: C3435T, C1236T and G2677T/A. These SNPs correlate by linkage disequilibrium, leading to the two most common haplotypes (1236C-2677G-3435C and 1236T-2677T-3435T) that are thought to be related to P-gp expression and function by several mechanisms. One of these mechanisms refers to the decrease of drug transport ability with the existence of dysfunctional transporters that are unable to bind to a substrate. These SNPs interfere in gene sequence, resulting in a protein with reduced binding ability. The studies that have been published to date with regard to the relationship between SNP and level of P-gp expression show contrasting results, suggesting that polymorphisms may interfere with response to chemotherapy treatment but fail to affect the outcome [10,13-17].

Hematological malignancies constitute a group of heterogeneous diseases that originate in a single pluripotent or differentiated hematopoietic cell. This heterogeneity reflects in biological and genetic features of the tumor, in the form of different clinical characteristics that limit multiple treatment options and prognostic variability.

Non-Hodgkin's lymphoma (NHL) is the most frequent hematological malignancy. According to the European Cancer Observatory, 49,533 new cases have been registered all over Europe in 2012 [18]. In the same period of time, 20,328 deaths have been attributed to this condition. The definition of 'NHL' comprises an extremely heterogeneous group of diseases that can be classified, according to their histopathological and genetic features, into 48 subtypes (27 of B-cell origin), with very different prognoses and outcomes [19].

2. Methods

A literature search was conducted using the PubMed database. The search terms used were: lymphoma, non-Hodgkin lymphoma, drug resistance, *MDR1*, multidrug resistance gene and P-glycoprotein. Only articles using human subjects were selected. Clinical case reports were excluded.

3. An overview of the implications of P-gp/ *MDR1* gene on NHL

3.1 Influence of P-gp expression

3.1.1 Effect on disease risk

P-gp is widely expressed in different tissues even in the absence of disease. Significant levels of P-gp expression are found in epithelial tissues, including but not limited to those with secretory or excretory functions [7]. P-gp expression in hematopoietic tissue can be explained by the protection function against external toxic exposures, for example, to pesticides [20], that can correlate with disease onset.

Different studies, by different working groups, show that P-gp expression is altered even before the disease is diagnosed. In 1995, a study involving 60 cases of acute leukemia and lymphoma was reported [21]; the authors concluded that an association can be established between P-gp expression and the T-phenotype – lymphoma the form of proliferation – but with a higher risk for development of drug resistance.

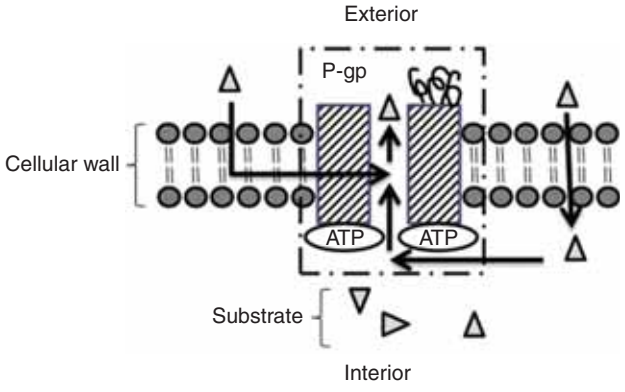

Figure 1. Schematic representation of the structure of P-glycoprotein (P-gp), its position on the cell membrane, the functional mechanism and its interaction with substrate.

The expression of P-gp prior to disease treatment was supported by a study published in 2001 using samples of B-cell NHL at diagnosis and relapse [22]. Furthermore, this result was confirmed in 2007 in patients with AIDS-related NHL [23]. However, more recently, in 2009, a study conducted by Szczuraszek *et al.* found that P-gp was not expressed in cases of non-treated NHL [24].

The expression of this particular protein was also investigated in samples of specific NHL subtypes. In a study of selected cases of cutaneous NHL, conducted by the Dutch Cutaneous Lymphoma Working Group, it was found that P-gp expression and disease phenotype are related and that both benign and lymphomatous skin disorders can overexpress P-gp [25]. A rare and aggressive subtype of lymphoma – the NK/T NHL – was studied by two different groups and their results showed high expression of P-gp in these tumors [26,27].

Higher P-gp expression can be related to the risk for development of NHL because it is found in clonal lymphoid tissue prior to treatment and even in premalignant conditions.

3.1.2 *Effect on outcome*

The great majority of studies related to P-gp expression and outcome in NHL indicate a correlation between high expression of P-gp and a poor prognosis of the disease. Older published studies debating this matter tend to show an absence of association with drug resistance and outcome. Until 1998, there were three main published studies, all of which failed to show significant linkage [28-30]. In a study published in 1992, Niehans *et al.* analyzed 57 samples of previously untreated diffuse large B-cell lymphoma (DLBCL) and immunoblastic lymphomas [28]. They concluded that P-gp immunoreactivity neither decreased the likelihood of response to induction chemotherapy nor adversely affected the median survival. Using the northern blot technique in samples from different tissues (reactive lymph nodes, tonsils and high-grade NHL), another study published in 1995 suggested that other explanatory reasons, besides P-gp/MDR, should be considered for disease resistance in NHL [24]. An additional study, published in 1998, aimed to find the relevance of P-gp expression in patients presenting with bcl-2 major breakpoint rearrangement, but no association was found between P-gp expression and disease prognosis [30]. More recently, Liu *et al.* were able to prove a significant relationship between high expression of *MDR1* gene and the probability of relapse or a poor prognosis in samples from patients with B-cell lymphomas [22].

Confirmatory results, using samples of newly diagnosed NHL, were reported in two separate studies published in 2006 and 2009, which confirmed the association between P-gp expression and poor prognosis and emphasized the important role that *MDR1* gene product overexpression may play in chemotherapy resistance [31,32].

A previously reported study in patients with nasal T-cell lymphoma concluded that the poor prognosis of this lymphoma subtype may be explained by the expression of P-gp [33]. A study restricted to DLBCL samples conducted in 2005 showed similar results in clinical outcome and prognosis [34]. A 1998 preliminary study of Burkitt lymphoma cell lines [35] concluded that the level of P-gp expression not only alters drug resistance but is also implicated in several other mechanisms involving migratory and adhesive properties. The same approach was adopted in cases of AIDS-related NHL, yielding similar results and demonstrating the role of P-gp expression in drug resistance [36,37]. In 2002, Tulpule *et al.*, using biopsy samples at diagnosis, showed that the level of P-gp expression was related to time to progression (TTP) [36]. Later, in 2005, the same group confirmed the relationship between P-gp expression and poor prognosis in this specific lymphoma subtype [37]. Another study by Tanaka and Calore reinforced this finding, and deduced from their work the association between this mechanism of drug resistance, treatment response and overall survival (OS) [23].

By combining the results of these studies, it is evident that there is a relationship between P-gp overexpression and prognosis. The discrepancies found between older and newer publications can be attributed to the different detection methodologies used.

3.2 Influence of *MDR1* polymorphisms on NHL

3.2.1 *Effect on disease risk*

The role of genetic risk factors in hematological malignancies is still not clarified, when compared with solid malignancies. The discovery of genetic hallmarks is the main goal of research aiming for the prevention and were definition of therapeutic targets.

In NHL cases, no relationship was observed between *MDR1* genetic polymorphism and disease development [38].

3.2.2 Effect on outcome

No significant association between C1236T SNP *MDR1* polymorphisms, drug resistance and even clinical outcome was reported in NHL [39].

A relevant link was established for NHL between G2677T/A genetic polymorphism and clinical outcome. This polymorphism also has a higher relevance because it is considered as an independent prognostic biomarker for OS, with a dominant combined effect of G2677T/A and C3435T specifically in DLBCL cases. In this NHL subtype, the presence of a T-carrier genotype G2677T/A has been associated with poor survival rates and higher drug resistance [40].

The C3435T polymorphism, besides its known effect on OS of DLBCL cases, in association with the G2677T/A polymorphism has also been proposed to be a determinant of the efficacy of all NHL chemotherapy schemes [38]. Although no relationship was found in the literature between polymorphisms and the risk of NHL development, an important association was found with outcome.

4. Reversing P-gp overexpression: implications of pharmacogenetic approaches in NHL

Some of the strategies used to overcome to overcome drug resistance are summarized on **Table 1**, in addition to the results of some clinical trials; these may act in several different ways: changing P-gp expression (e.g., benzofuran) [41], removing P-gp from the dual lipid layer (e.g., rituximab) [42], competing with the substrate for binding affinity (e.g., ritonavir and saquinavir) [43,44], increasing apoptosis in cells showing high P-gp expression (e.g., cyclosporine, valspodar) [45,46] or covering the drug, leading to its non-recognition as a substrate by P-gpand thereby evading the efflux pump (e.g., liposomal non-pegylated doxorubicin) [47-49]. A schematic representation of these different strategies can be found in **Figure 2**.

One interesting clinical result is the use of liposomal non-pegylated doxorubicin. Anthracyclines (like doxorubicin) have proven their importance in NHL treatment. By contrast, it is a substrate for P-gp, meaning that the interface with this protein can lead to less efficacy. The lipid formulation of anthracyclines promotes its interiorization within the cell, thereby overcoming the efflux pump and thus yields additional results [47-49].

In vitro assays using resistant cell lines, aiming to achieve chemosensibilization with monoclonal antibodies, were also tried with both anti-CD19 and anti-CD20. These two antibodies can change the P-gp localization by removing it from the cell membrane, and and therefore lead to a decrease in its function [42,50].

Cases of AIDS-related NHL are known for their disappointing clinical results in spite of many efforts for their treatment. Studies conducted associating both chemotherapy regimens and antiviral therapies with ritonavir and saquinavir suggest the potential of these drugs to help regain cell susceptibility to chemotherapy [44]. These drugs act as genuine P-gp substrates by inhibiting dye substrate efflux and reversing drug resistance [43].

Some drugs such as bortezomib, valspodar, tamoxifen and cyclosporine are also capable of inhibiting ceramide metabolism, inducing cellular apoptosis [46,51-67]. Perifosine is an Akt inhibitor that induces cellular death; it is also implicated in decreased P-gp mRNA expression [68-71]. Quinine, another drug that is commonly used for other purposes, is also related to decreased P-gp expression, with implications for reverting drug resistance [72-76].

Zosuquidar, a third-generation P-gp inhibitor, is a potent and highly selective P-gp modulator [77-83]. Security studies demonstrated that it had a minimal effect on the pharmacokinetic profile of coadministered P-gp substrates and that it does not inhibit other members of the ABC transporter family or the function of cytochrome p450.

Other different therapeutic options have been tried, including molecular techniques aiming to reverse P-gp overexpression using ribosomes of *MDR1* negative cells transfected into the P-gp overexpressed cells [84].

The sensitivity and specificity of a pharmacogenetic profile including MDR genetic variants are to be taken in perspective based on the different agents involved in disease treatment. All the above strategies for reversing P-gp overexpression/function (**Figure 2**) have future implications for pharmacogenetic approaches to NHL therapy due to their influence on clinical outcome.

Nevertheless, until now, there is published evidence only for cyclosporine overcoming drug resistance, which has shown benefit in TTP with its addition to common chemotherapy in patients with acute myeloid leukemia [66].

The main issue when modifying P-gp expression is the likelihood of interfering with physiological pathways, because this protein is widely expressed in varying concentrations in almost every tissue [7,8]. With the need for increasing cellular drug susceptibility, many drugs have been tried but some have never passed the test of toxicity. Cyclosporine was the first drug to pass both efficacy and security tests [66,85-87], but other so-called 'second-generation inhibitors' failed to do so. Valspodar is one of the drugs with accepted tolerability [59,63]. 'Third-generation inhibitors'were designed with the aim of trying to improve responses with minimal side effects. Zosuquidar is one of these third-generation inhibitors with selectivity to P-gp and minimal systemic side effects [77,81,82].

5. Conclusion

It is known that P-gp is widely expressed in human tissues with putative defensive functions (liver, kidney and intestinal mucosa), and is uncommon yet still present in hematopoietic cells, where the expression is significantly lower. P-gp overexpression, as a *MDR1* gene product, was found in both

Table 1. Possible therapeutic interventions, aiming at reversal of MDR incases of NHL and acute leukemia.

Therapeutic intervention	Mechanism of action	Refs.
Benzofuran	Of the 24 compounds some showed good antiproliferative activity while others exhibited good MDR reversal activity	[41]
Liposomal non-pegylated doxorubicin	Liposomal encapsulation might evade resistance caused by *MDR1* expression	[47-49]
Rituximab	The inhibition of P-gp activity correlated with the ability of rituximab to induce P-gp to translocate out of lipid rafts	[42]
Monoclonal antibody anti-CD19	These results suggest that anti-CD19 might chemosensitize P-gp(+) cells by interfering with interactions between CD19 and P-gp, resulting in the rapid translocation of P-gp into a compartment on the plasma membrane where it is no longer active	[50]
Ritonavir and saquinavir	These protease inhibitors may render P-gp expressing MDR cells *de novo* susceptible to the antineoplastic drugs vinblastine, vincristine and doxorubicin	[43-44]
Bortezomib	Potentially inhibited growth and increased the apoptosis rate in cell lines Bortezomib and daunorubicin have a synergistic effect on antiproliferation	[51-57]
Valspodar	Non-immunosuppressive analog of cyclosporine Potent P-gp inhibitor Modulates ceramide metabolism, which is important in apoptosis Can potentiate apoptosis induced by some anticancer agents	[46, 59-63]
Tamoxifen	Increases intracellular daunorubicin by inhibiting P-gp efflux function Modulates ceramide metabolism, which is important in apoptosis	[58]
Cyclosporine	Modulates ceramide metabolism, which is important in apoptosis	[45, 64-67, 85-87]
Perifosine	Akt inhibitor Induces death by apoptosis, interacting with the integrity of lipid rafts Downloads the expression of P-gp mRNA	[68-71]
Zosuquidar	Inhibition of P-gp in the bile duct canaliculi impeding biliary excretion	[77-83]
Quinine	P-gp reversing activity	[72-76]
Ribosomes	Ribosomes of *MDR1* negative cells are transfected into the cell that overexpress P-gp with reversion of the expression	[84]

MDR: Multidrug resistance; NHL: Non-Hodgkin's lymphoma; P-gp: P-glycoprotein.

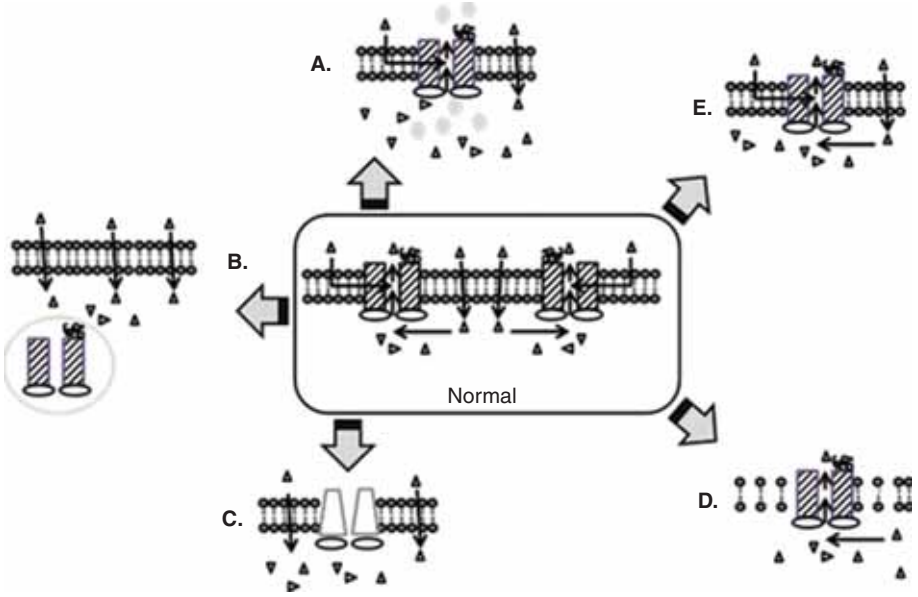

Figure 2. Schematic representation of the modulation mechanisms of P-gp. (**A**) Inhibition by another P-gp substrate (e.g., quinine), (**B**) interiorization of the protein (e.g., rituximab), (**C**) protein folding alteration by single nucleotide polymorphism, (**D**) apoptosis (e.g., bortezomib), and (**E**) decreased mRNA with decreased P-gp expression (e.g., perifosine).

malignant and non-malignant tissues, which can explain why some benign conditions may transform into clonal disorders. In NHL, this expression occurs prior to treatment, which means that higher rates of overexpression incases of relapse may result from specific clone selection in disease progression.

P-gp expression can be related to *MDR1* gene SNP, with published work showing a relationship between some haplotypes and protein expression. P-gp overexpression can be linked to the clinical outcome. This is consistently observed in several different studies, in particular lymphoma subtypes, showing statistically significant differences in OS, risk of relapse and duration of response. It has been proposed that *MDR1* polymorphisms are related to the expression of P-gp, confining genetic sequence alteration that can change the substrate binding and protein function. These polymorphisms have been implicated in the risk of childhood acute lymphoblastic leukemia. Regarding clinical outcome, *MDR1* gene SNPs have been associated with response to induction chemotherapy, risk of relapse, TTP and OS.

Much effort has been made in the attempt to pharmacologically overcome drug resistance using already known compounds or by designing new ones. However, some discrepancies can be found in outcome results with the association of cyclosporine to conventional chemotherapy; this drug has proven efficacy, resulting in prolonged TTP in specific cases. The other P-gp inhibitors, which were designed based on cyclosporine, do not improve previously achieved results and lacked acceptable safety profiles because they failed in terms of the specificity: inhibition of *MDR1* gene and *MRP1* gene along with pharmacokinetic interactions because they interfere with cytochrome p450. The newest drug in this field, zosuquidar, is an *MDR1* gene selective inhibitor, which is more efficient and less toxic. However, modulating *MDR1* gene-related drug resistance is 'an old story'; many are still not done and there is a long way to go: new drugs are needed, with consistent outcomes and minimum side effects. For this, we need to get a good understanding of the genetic causes for drug resistance of cancer cells.

6. Expert opinion

Since 1976, when the *MDR1* gene was first described, *MDR1* along with its protein, P-gp, has been at the center of many pharmacogenomic approaches. Most of the development concerning treatment of NHL was achieved in the past decades, mainly with the use of monoclonal antibodies that target disease.

Treatment failure is still the most concerning cause of disease progression and cancer-related death, which is commonly related to therapy resistance. The product of the *MDR1* gene (P-gp) has been related to this particular cause of therapeutic failure. On the basis of different studies conducted over several years, a relationship with outcome was established. Data also suggest interference with risk of NHL development based on high P-gp expression level in premalignant conditions and in malignant disease prior to treatment, which means that it precedes clonal differentiation and does not result from treatment.

Some *MDR1* gene polymorphisms correlate with increased levels of P-gp expression. Relationships between some of these SNPs (C2425T and G2677T) and outcome were also observed. These two polymorphisms have also been considered as independent prognostic biomarkers for DLBCL. No relationship was found with the risk of NHL development.

The implications of P-gp overexpression in on outcome have made this protein a very integrating therapeutic target. Cyclosporine was the first P-gp inhibitor that, to date, was the most used and the best tolerated with minimum side effects. Attempts were made to create a better molecule that reproduces the success of cyclosporine along with an improved toxicity profile. These 'second-generation inhibitors' were synthesized but they failed especially in the toxicity profile. The main reason for this is the decreased specificity for MDR1 by the inhibitors created, which can bind not only to MDR1 but also to other transporters of the ABC transporter family. These drugs can also affect cytochrome p450, influencing the metabolization of many compounds commonly used in chemotherapy regimens. Zosuquidar is a recent P-gp inhibitor that preferentially binds to the *MDR1* gene transporter with a reduced toxicity profile.

Other therapeutic options have been used in trying to mask the effect of P-gp, with limited success in some clinical trials, but mostly in tumor cell lines or in a small number of cases.

Decades after the discovery of the *MDR1* gene and its correlation with drug resistance, there is still much research to be done. Strategies to overcome P-gp-mediated drug resistance need to have a less aggressive toxicological profile with fewer drug interactions, permitting its use in a broader spectrum of patients. Specificity of inhibition affecting only the *MDR1* gene and P-gp will allow a more efficient and less toxic combination with the current chemotherapy schemes improving outcome.

Declaration of interest

The authors state no conflict of interest.

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We conducted a study analysing 175 Portuguese Acute Leukemia and Non-Hodgkin Lymphoma patients. To validate our results we analysed the relation between genotype expression and disease (AL an NHL) incidence and prevalence rates in several populations.
We observed that C1236T polymorphism influences risk for Acute Leukemia development in the Portuguese population (T vs C: p= 0,026 OR 0,49 95CI 0,24-0,97; CC vs TT: p= 0,030 OR 7,37 95CI 0,94-156,8). When we analysed a group of European and Asian populations we found a significant correlation between the incidence and prevalence rates and CC genotype expression.
We conclude that MDR1 C1236T polymorphism influences risk for leukemia development perhaps because it interferes with protein folding and substrate binding capacity. The disability of extrude compounds from the inside of the cell may lead to the toxic influence of some compounds, contributing to its leukemogenic effect.

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Multidrug Resistance Gene C1236T polymorphism influences risk for acute leukemia

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Introduction

The human Multidrug Resistance Gene 1 (MDR 1 or ABCB 1) was isolated in 1986 corresponding to the genetic location at chromosome 7 (q21.1) of the P-glycoprotein, [1-3], that has been isolated 10 years earlier from hamster ovary cells [4, 5]. This protein is phosphorylated and glycosylated, with 1280 amino acids, with a molecular weight of 170 kDa. It is composed of two homologous halves, each one containing six putative transmembrane domains and an intracellular ATP binding site. P-glycoprotein (p-gp) function is to extrude several endogenous and exogenous substances using the ATP-dependent pump [6-10], carrying the substrate from the interior leaflet to the exterior. The association of p-gp with intrinsic drug resistance to cancer therapy is based on the ability of exporting unnecessary and toxic exogenous substances or metabolites out of the body. This ability is associated with a cellular defence mechanism and when is altered can lead to the development of cancer[11], like colon cancer [12], hepatocarcinoma [13], acute lymphoblastic leukemia[7, 14] and gall bladder tumours [15] . The dysfunction of this protein was also involved in risk for other diseases and also in the therapeutic response, in cases like epilepsy [16], major depression disorder [17] AIDS [18], malaria [19] and inflammatory bowel disease [20]. It is widely expressed in almost all human tissue (liver, kidney, small and large bowel, brain, testis, muscle, placenta and adrenals) where it mediates the efflux of xenobiotics [21, 22].

Single nucleotide polymorphisms (SNP) are the most frequently inherited sequence variations in a particular gene. Since 1989 several polymorphisms has been described in this gene [23]. Until today more than 50 have been identified [24-27]. Among all this defined polymorphisms, 3 are known to occur frequently (>0,1) in various populations: C3435T, C1236T and G2677T/A. These SNP correlate by linkage disequilibrium, originating the 2 most common haplotypes (1236C – 2677G – 3435C and 1236T

– 2677T – 3435T) thought to be related to P-gp function [28]. The genotype is probably associated with protein (P-gp) folding, changing substrate specificity and its expression [24, 29-32].

Acute leukemias (myeloid or lymphoid) are haematological malignancies, characterized by undifferentiated and uncontrolled proliferation of hematopoietic progenitor cells. Chemotherapy regimens contain several cytotoxic compounds, some in high dose regimens, aiming to achieve response and cure, overcoming primary drug resistance. In acute myeloid leukemia (AML) cases patients do reach primary response, but almost 70% relapse and 80% die from leukemia. In adult acute lymphoblastic leukemia (ALL) responses are inferior to the myeloid counterpart.

Non-Hodgkin Lymphoma (NHL) are a heterogeneous group of haematological malignancies that consist of the clonal differentiation of a mature lymphoid cell (B, T or NK origin). This definition includes several subtypes with different aetiology, treatment and prognosis. They can be divided in 2 major groups: the aggressive and the indolent lymphomas. The most common subtype, the Diffuse Large B cell Lymphoma (DLBC), an aggressive lymphoma. The common standard of care includes R-CHOP (rituximab, cyclophosphamide, vincristine, doxorubicin and prednisolone) with an Overall survival (OS) at 10 years of 80%. In the indolent counterpart the Follicular Lymphoma (FL) is the most frequent. The current standard of care is also R-CHOP regimens but "watch and wait" or radiotherapy can also be treatment options based on stage disease and patient symptoms. The natural disease history of this particular condition consists with several relapses, ranging (OS) at 10 years from 40 to 85%.

The aim of our study was to evaluate the influence of MDR1 associated polymorphisms genetic background in the susceptibility to AL and NHL development.

Materials and methods

A total of 175 patients were studied: 37 with acute leukemia (AL) diagnosis (22 with acute myeloid leukemia) and 138 with Non Hodgkin Lymphoma (NHL). Patient data was collected from clinical electronic file after signed informed consent.

Healthy control individuals were recruited among IPO-Porto blood donors population. These controls consisted of 160 healthy individuals, 118 females, with median age of 41 years.

Peripheral blood samples in EDTA containing tubes were collected by common venepuncture. The genomic DNA was extracted from the whole blood using a commercial kit (E.Z.N.A. – Omega Bio-Teck, Norcross, USA) according to the manufacturer's instructions and stored at -20ºC.

The polymorphisms C3435T and C1236T were genotyped by Real Time PCR (RT-PCR) with Taqman®SNP Assay genotyping C___7586857_20 and C___7586662_10, respectively, from Applied Biosystems®. Genotyping data were analysed blind to the clinical course. In case of dubious genotyping result the study was repeated.

Statistical analysis. Analysis of data was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 17.0). Differences in proportions were evaluated by the X^2 test. The probabilities of survival were calculated, and the means and life tables were computed using the

product limit estimate of Kaplan-Meier and analysed by the Breslow (generalized Wilcoxon) test, a statistical test for equality of survival distributions. A level of $p < 0.05$ was considered statistically significant.

The association between MDR1 genotype frequencies and disease (AL and NHL) incidence and prevalence variations among different populations was analysed using Pearson correlation coefficient. Incidence and prevalence of acute leukemia and non-Hodgkin lymphoma were accessed at http://globocan.iarc.fr. Incidence of genotypes of both C3435T and C1236T were obtained at http://www.appliedbiosystems.com.

This investigation is in accordance with the principle of the Declaration of Helsinki and was approved by the local Ethical Committee.

Results

A universe of 175 patients was studied, 37 with AL diagnosis and 138 with NHL. Acute leukemia patients were predominantly males (73%; n= 27), with a median age at diagnosis of 52 years (range 18 - 72). In the NHL group both sexes are equally represented with a slight male predominance (n= 70; 50,7%), with a median age at diagnosis of 59 years (range 18 – 88).

Two polymorphisms of the ABCB1gene were studied: C3435T and C1236T. The distribution of these polymorphisms in AL, NHL cases and controls are similar (Table 1). All cases are in Hardy-Weinberg equilibrium.

Regarding C3435T SNP in AL cases no significant differences were found between patients and control group. In C1236T polymorphism relevant differences in genotype and allele frequency are statistically relevant (table 2). In these cases the presence of an allele C represents a higher risk for disease (HR 0,49 95CI 0,24 – 0,97; p= 0,026), statistically relevant in CC genotype (HR 7,37 95CI 0,94 – 156,8; p= 0,03).

In NHL patients neither C3435T nor C1236T correlates with disease risk (table 3).

Using previously published data we analysed the effect of these 2 polymorphisms in risk and outcome for AL and NHL in a subset of European and Asian population (Table 4).

We observed variability in disease incidence (ASR/100000 habitants) that ranges from 0,7 in India to 20,1 in the UK for NHL and from 2,8 in India to 7,8 in Germany in AL cases.

The lowest prevalence (ASR/100000 habitants) of NHL is observed in India (1,5) and the highest in Germany (56,5). Acute leukemia is more prevalent in Poland (17,6) and less common in Japan (0,7).

The genotype frequencies distribution is also distinct among the studied populations. In C1236T polymorphism the CC genotype frequencies ranges from a minimum of 11% observed in the Japan and China to a maximum of 35,7% observed in Spain.

Regarding C3435T the variability of the CC genotype ranges from 17,6% observed in Iran to 30% in China.

When we studied disease incidence / prevalence and its association with genotype distribution we found a correlation between C1236T CC genotype frequencies and leukemia (Fig 1). This correlation was not present for NHL.

There was no correlation between C3435T genotype expression and disease incidence or prevalence (Fig 2).

Discussion

This study associates the CC genotype distribution in C1236T polymorphism with the risk of AL development. Our results are in concordance with previously reported results in other populations [33, 34].

Leukemogenesis is a multistep pathway where both environmental agents and individual genetic features interact. Since 1989 that multiple SNP have been identified in MDR1 gene and C3435T, C1236T and C2677T/A are the most frequently studied. Over the years many studies have been conducted regarding this subject but results are not conclusive. However, the scientific interest in this SNP is high due to the potential influence of these polymorphisms in disease risk, therapeutic success and outcome [32].

The C3435T polymorphism is the one more definitively associated with MDR1 expression and P-gp activity [8]. However, these SNPs act in linkage disequilibrium and their functional influence has been suggested. The results published suggest that the substitution to some rare codons can affect protein folding and insertion in the cell membrane, affecting substrate binding sites [31]. This alteration occurs when MDR1 gene is overexpressed. The dysfunction of the binding ability can explain the role of MDR1 polymorphisms in leukemogenesis [8, 22, 30, 31]. The cell toxicity of some compounds, like benzene and pesticides, can result from the incapacity of the cell to clean them from its cytoplasm, increasing disease risk [35-41].

Our results did not demonstrate the same association in risk to NHL. We may suggest that this can be explained because of the different nature of leukemia and lymphoma oncogenesis. In lymphoma development we are dealing with a non-apoptotic phenomenon leading to accumulation of a huge number of neoplasic cells. On the contrary in leukemia we are observing a phenomenon involving the pluripotent stem cell with all its abilities of self-renewing and proliferation. As this cells have a permanent intensive division rate are much more prone to the toxic effects of some compounds in DNA then others with a much lower division rate.

The discrepancies between different studies published may be explained by ethnic heterogeneity. As shown in the present study different populations have different genotype expressions. But even in the same ethnic group inconsistent results may be present because lifestyle, genetic background and toxic exposure differ, leading to different selective pressure and consequent different influences in cancer risk.

Conclusions

We analysed the expression of two common polymorphisms of ABCB1 gene, C1236T and C3435T, in a subset of Portuguese patients with the diagnosis of a haematological malignancy and in a control group of

healthy individuals. For the best of our knowledge this is the first work that correlates MDR1 polymorphism expression and risk of AL in the Portuguese population. We also proved the correlation between genotype expression (C1236T) and AL incidence and prevalence rates among other populations besides Portuguese. In NHL cases the genotype profile did not influenced disease rates.
More studies are needed analysing more patients to overcome ethnic bias aiming more consistent and validated results.

Author's contribution

All authors have contributed sufficiently to the project to be included as authors.

Compliance with Ethical Standards

The authors declare no conflict of interest regarding the publication of this paper.
This investigation is in accordance with the principle of the Declaration of Helsinki and was approved by the local Ethical Committee.
All patients signed an informed consent previously to samples collection.

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TABLES AND FIGURES LEGENDS:

Table 1: Allele / genotype frequency and Hardy-Weinberg equilibrium in both studied polymorphisms in controls, AL and NHL population

Table 2: Allele and genotype frequencies of ABCB1ABCB1 gene polymorphisms and risk for AL

Table 3: Allele and genotype frequencies of ABCB1 gene polymorphisms and risk for NHL

Table 4: Different incidence, prevalence and mortality in acute leukemia a Non-Hodgkin lymphoma cases among different populations (Incidence, prevalence and mortality expressed in ASR – age specific rates/ 100 000 habitants; genotype frequencies expressed in %)

Fig. 1: Graphic correlation of polymorphism C1236T with both incidence and prevalence of acute leukemia and non-Hodgkin lymphoma (correlation coefficient is as follows: A-0,617 B-0,361 C-0,679 D-0,307)

Fig 2: Graphic correlation of polymorphism C3435T with both incidence and prevalence of acute leukemia and non-Hodgkin lymphoma (correlation coefficient is as follows: A-0,262 B-0,007 C-0,057 D-0,024)

Figure 1

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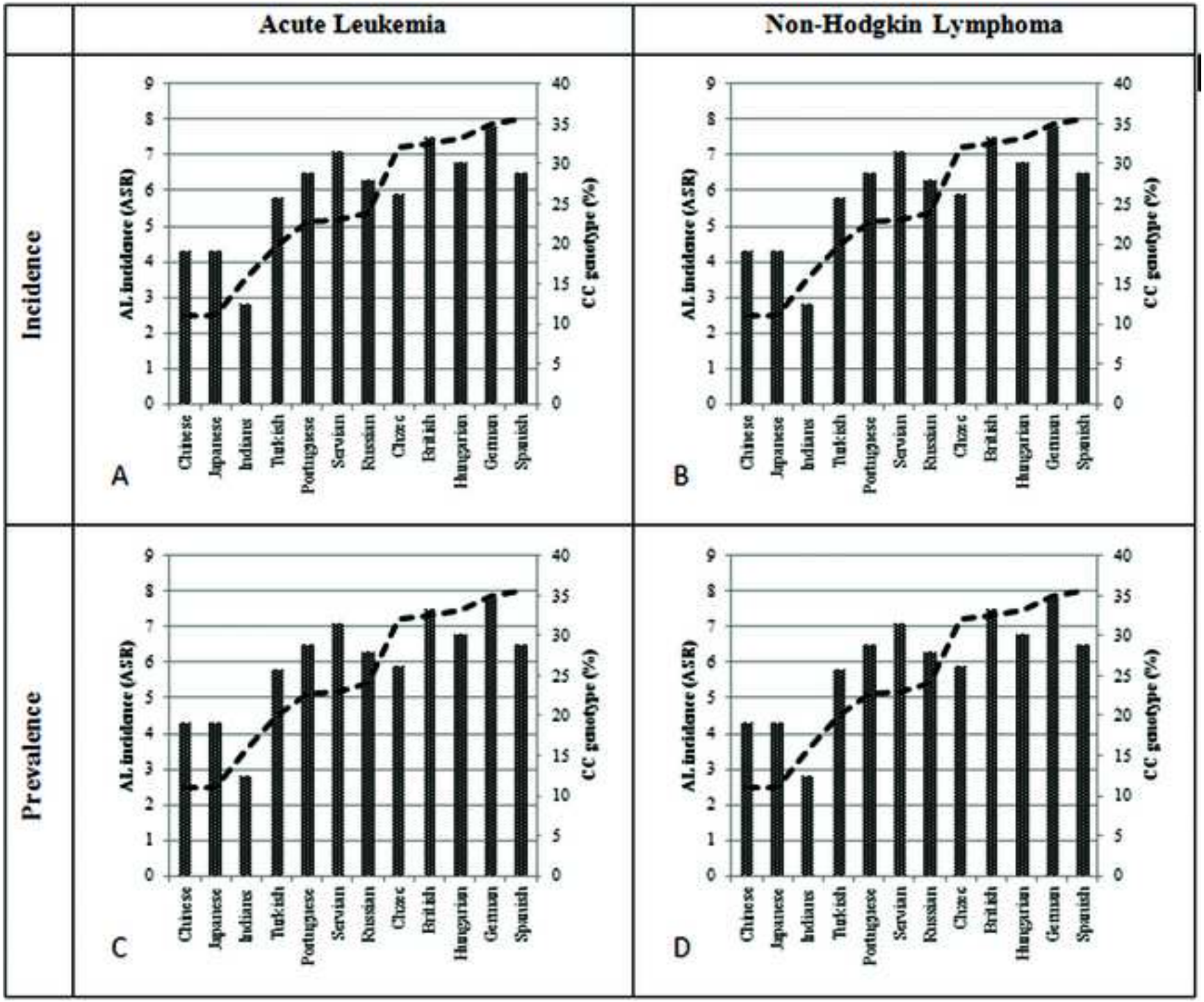

Figure 2

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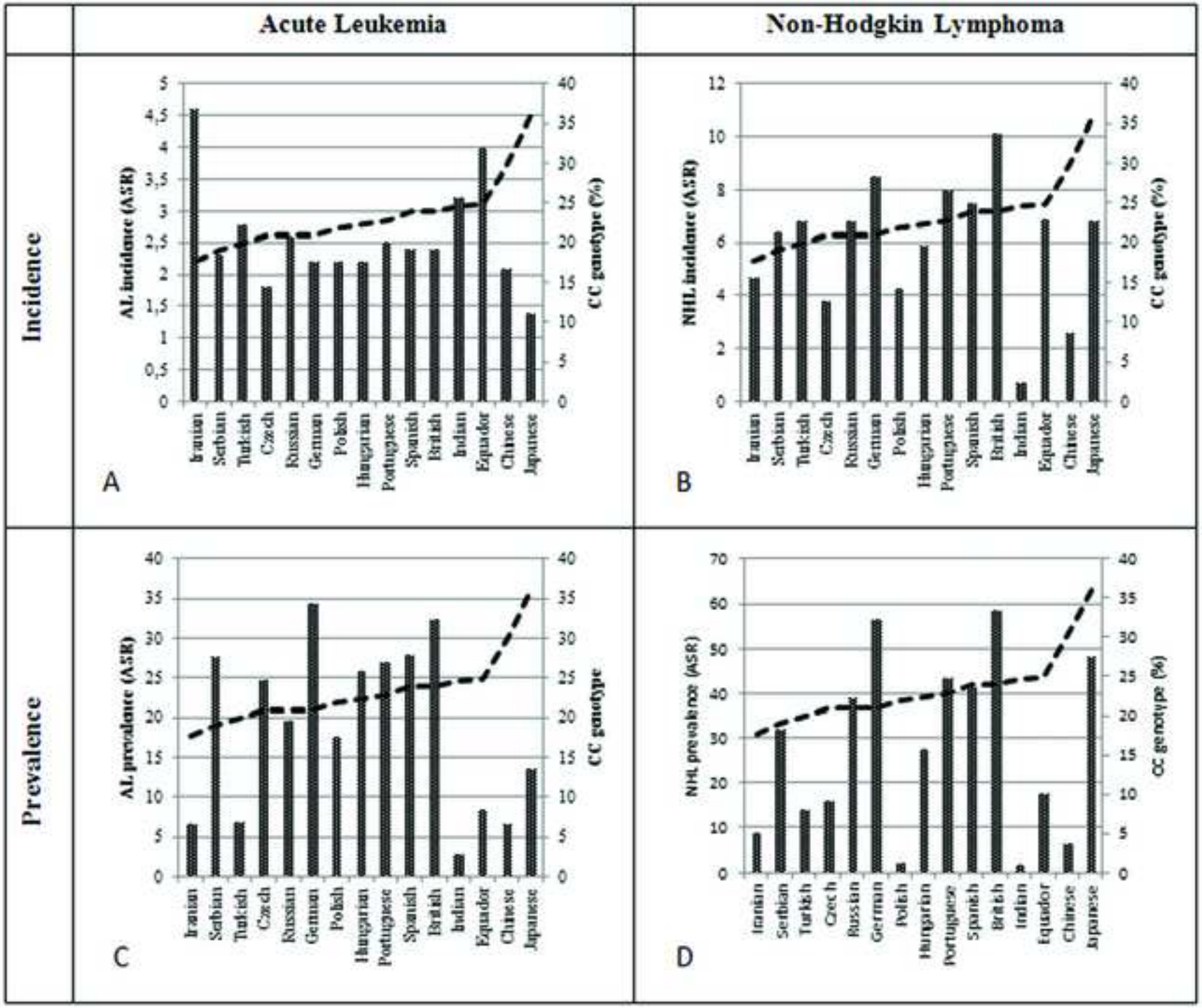

Table 1

Table 1: Allele / genotype frequency and Hardy-Weinberg equilibrium in both studied polymorphisms in controls, AL and NHL population

		AL		NHL		Controls		HWE *p*		
		n	**%**	**n**	**%**	**n**	**%**	**AL**	**NHL**	**Controls**
C3435T	**Allele**									
	C	39	65	152	55,1	185	57,8	0,178	0,154	0,413
	T	21	35	124	44,9	135	42,2			
	Genotype									
	CC	11	36,7	46	33,3	56	35			
	CT	17	56,7	60	43,5	73	45,6			
	TT	2	6,7	32	23,2	31	19,4			
	C carriers	28	93,3	106	76,8	129	80,6			
C1236T	**Allele**									
	C	42	75	111	55	190	59,4	0,449	0,160	0,395
	T	14	25	91	45	130	40,6			
	Genotype									
	CC	15	53,6	27	26,7	59	36,9			
	CT	12	42,9	57	56,4	72	45			
	TT	1	3,6	17	17	29	18,1			
	C carriers	27	96,4	84	83,2	131	81,9			

Table 2

Table 2: Allele and genotype frequencies of ABCB1ABCB1 gene polymorphisms and risk for AL

Polymorphism		Controls	n (AL)	OR (95% CI)	*p*
C3435T	**Allele**				
	C	185	39	*Reference*	
	T	135	21	1,36 (0,74 – 2,51)	0,300
	Genotype				
	TT	31	2	*Reference*	
	CT	73	17	0,33 (0,05 – 1,73)	0,148
	CC	56	11	0,28 (0,04 – 1,379	0,811
	C carriers	129	28	3,36 (0,72 – 21,6)	0,092
C1236T	**Allele**				
	C	190	42	*Reference*	
	T	130	14	0,49 (0,24 – 0,97)	**0,026***
	Genotype				
	TT	29	1	*Reference*	
	CT	72	12	4,83 (0,60 – 103,96)	0,105
	CC	59	15	7,37 (0,94 – 156,8)	**0,030***
	C carriers	131	27	5,98 (0,81 – 122,85)	0,523

Table 3

Table 3: Allele and genotype frequencies of ABCB1 gene polymorphisms and risk for NHL

Polymorphism		Controls	n (NHL)	OR (95% CI)	*p*
C3435T	**Allele**				
	C	185	152	*Reference*	
	T	135	124	0,89 (0,64 – 1,25)	0,501
	Genotype				
	TT	31	32	*Reference*	
	CT	73	60	1,26 (0,66 – 2,39)	0,457
	CC	56	46	1,26 (0,64 – 2,48)	0,476
	C carriers	129	106	0,80 (0,44 – 1,44)	0,421
C1236T	**Allele**				
	C	190	111	*Reference*	
	T	130	91	0,83 (0,58 – 1,21)	0,320
	Genotype				
	TT	29	17	*Reference*	
	CT	72	57	0,74 (0,35 – 1,56)	0,394
	CC	59	27	1,28 (0,56 – 2,90)	0,519
	C carriers	131	84	1,09 (0,54 – 2,23)	0,789

Table 4

Table 4: Different incidence, prevalence and mortality in acute leukemia a Non-Hodgkin lymphoma cases among different populations (Incidence, prevalence and mortality expressed in ASR – age specific rates/ 100 000 habitants; genotype frequencies expressed in %)

Population	Non-Hodgkin Lymphoma			Acute Leukemia			CC Genotype	
	Incidence	Prevalence	Mortality	Incidence	Prevalence	Mortality	C1236T	C3435T
Chinese	2,60	6,30	1,60	4,30	1,50	3,60	11,00	30,00
Czech	6,80	38,90	2,20	5,90	1,50	3,40	32,00	21,00
Equador	6,90	17,5	3,2	6,5	8,40	5,00	-	24,90
German	8,50	56,50	2,30	7,80	1,80	3,30	35,00	21,00
Hungarian	5,90	27,50	2,50	6,80	1,90	4,00	33,20	22,30
Indians	0,70	1,50	0,40	2,80	1,30	2,30	15,60	24,70
Iran	5,80	8,8	3,0	5,8	6,7	4,6	-	17,60
Japanese	6,80	48,30	2,60	4,30	0,70	2,70	11,00	36,00
Polish	5,60	2,0	2,3	5,6	17,6	3,8	-	22,00
Portuguese	8,00	43,50	3,10	6,50	2,10	3,70	22,80	22,80
Russian	3,80	16,10	1,90	6,30	2,20	3,40	24,00	21,00
Serbian	6,40	31,80	2,80	7,10	2,00	4,60	23,00	19,00
Spanish	7,50	41,20	2,20	6,50	1,90	3,00	35,70	24,00
Turkish	6,80	13,90	4,30	5,80	1,20	4,60	20,00	20,00
UK (caucasian)	10,10	58,60	2,90	7,50	2,00	3,20	32,60	24,00